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Response of oligotrophic coastal microbial populations in the SE Mediterranean Sea to crude oil pollution; lessons from mesocosm studies

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ABSTRACT

Anthropogenically-induced oil spills release large amounts of organic pollutants into the marine environment. To date, little is known about the response of microbial populations (biomass, activity and diversity) to crude oil pollution in Low Nutrients Low Chlorophyll and warm systems. Here, we investigated the daily dynamics of phytoplankton and heterotrophic bacteria in response to an oil spill (500 μm thick layer) in the coastal waters of the SE Mediterranean Sea (SEMS), using mesocosms during winter and summer. Crude oil addition caused a marked decrease in phytoplankton biomass (40–76%) and production rates (22–96%), whereas heterotrophic bacterial abundance and production increased (4–68% and 17–165%, respectively). Concurrently, amplicon sequencing of the 16S rRNA gene revealed that oil-degrading bacteria became abundant 48–96 h post-oil addition, while the cosmopolitan *Synechococcus* and SAR11 lineages were significantly reduced (by 78–98% and 59–98%, respectively). Fertilization with inorganic nutrients (NO_3 and PO_4) reduced the deleterious effects of the oil, resulting in a less distinct reduction in phytoplankton biomass/abundance. Our results highlight the potential of intrinsic microbial communities to degrade oil-derived pollutants in oligotrophic coastal waters.

1. Introduction

A large proportion of the global marine oil used is transported across the Mediterranean Sea (20%–25%), and millions of tons of petroleum and its byproducts are moved annually to/from the southeastern Mediterranean Sea (SEMS) (Amir, 2019). Occasional spills of these transported hydrocarbons as a result of accidents, failures, heavy weather, damage and human error (ITOPF, 2019) may have pronounced effects on marine life on short (hours to days) to long (months to years) term timescales. Recent years have shown a major increase in the number of offshore oil and gas exploration and production activities in the eastern Mediterranean sea, increasing the risk of a major oil spill event (Brenner, 2019).

The organisms that are likely to be affected first by oil spill pollution are sea-surface or coastal microbial populations (Douglas et al., 1994; Gros et al., 2014; Brussaard et al., 2016). Several studies showed that oil spills may lead to a decline in algal biomass and primary productivity (González et al., 2009; Gilde et al., 2012), as well as alterations of heterotrophic bacterial abundance (Kimes et al., 2014; Chronopoulou et al., 2015; Brussaard et al., 2016) and function (Nayar et al., 2005; Gertler et al., 2012; Kimes et al., 2014). Such changes are likely to impact the marine food web and favor specific microbial populations capable of using oil-derived hydrocarbons (e.g., *Alcanivorax* sp., *Glaciecola* sp., *Pseudoalteromonas* sp., *Oceanospirillales* sp.; (Hazen et al., 2010; Tremblay et al., 2019; Chronopoulou et al., 2015), thus potentially leading to changes in ecosystem health (Brussaard et al., 2016).

The coastal SEMS water is warm throughout the year (temperature range 17–31 °C, Rilov, 2016), and has Low Nutrients Low Chlorophyll (LNLC) characteristics (Raveh et al., 2015; Kress et al., 2019; Rahav et al., 2018a). The SEMS microbial community consists mainly of small cyanobacteria and heterotrophic bacteria (Rahav et al., 2016; Rahav and Bar-Zeev, 2017; Yacobi et al., 1991) (and primary production is usually low (Rahav et al., 2018b; Frank et al., 2017)). Due to these oligotrophic characteristics, the SEMS is considered a 'sensitive' environment where

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external anthropogenic nutrient inputs are likely to stimulate microbial activity and alter key features in the marine ecosystem. This is especially true if these waters are enriched in NO_3 (Rahav et al., 2018a, 2018b; Rahav et al., 2018a; Raveh et al., 2019), NO_3+PO_4 (Kress et al., 2005) and/or organic carbon/phosphorus (Rahav et al., 2016; Sisma-Ventura and Rahav, 2019), as these are known to limit phytoplankton in the coastal waters of the SEMS.

Here, we investigated how coastal surface-water microbial populations of the SEMS would respond to crude-oil (organic carbon) addition. To this end, two mesocosm experiments were conducted to test the response of autotrophic and heterotrophic microbial populations to oil pollution. We also examined whether the amendments of the potentially limiting nutrients, NO_3 and PO_4 in addition to the crude-oil pollution would affect the populations of the planktonic microbes. We hypothesized that due to the oligotrophic nature of SEMS water, crude-oil addition would cause major changes in cyanobacteria, phytoplankton and heterotrophic bacterial biomass, activity and diversity, whereas the addition of nutrients should accelerate oil degradation rate by stimulating local bacterial oil consumers. We also hypothesized that the addition of limiting nutrients such as NO_3 and PO_4 would reduce the deleterious effects of the crude-oil on phytoplankton.

2. Material and methods

2.1. Experimental setup

Two mesocosm experiments were conducted: one during midwinter (December 2017) and one during midsummer (July 2018). Nine transparent polyethylene mesocosm bags, 1 m^3 each, were placed in a 16 m^3 concrete pool, at the Israel Oceanographic & Limnological Research outdoor experimental facility with continuously flowing seawater pumped into the concrete pools from the nearby shore, to maintain ambient seawater temperature (Fig. 1). Each mesocosm bag was filled with surface (~1 m) SEMS coastal water (natural populations, no pre-filtration step was carried out). Two crude-oil amendments and one control were set up in triplicate mesocosms: [1] no addition (control); [2] addition of 565 mL light crude-oil from the Haifa refinery to form a 500 μm thick layer at the water surface, which corresponds to a heavy pollution scenario based on the Israeli Ministry of Environmental Protection (www.sviva.gov.il); [3] crude-oil (prepared as above) + NO_3 (1600 nM) and PO_4 (100 nM). While a 500 μm thick cruse-oil layer may result in reduced light attenuation, due to the transparency of the mesocosm bags (Fig. 1), it is unlikely that any light limitation had occurred. Sub-samples were removed from the mesocosms daily at 09:00 a.m. using Tygon tubes in T-shaped pipes to ensure that samples were taken from ~50 cm above the mesocosm bottom. The experiments lasted 5–8 days (winter and summer, respectively) and samples were taken for a suite of measurements as described below.

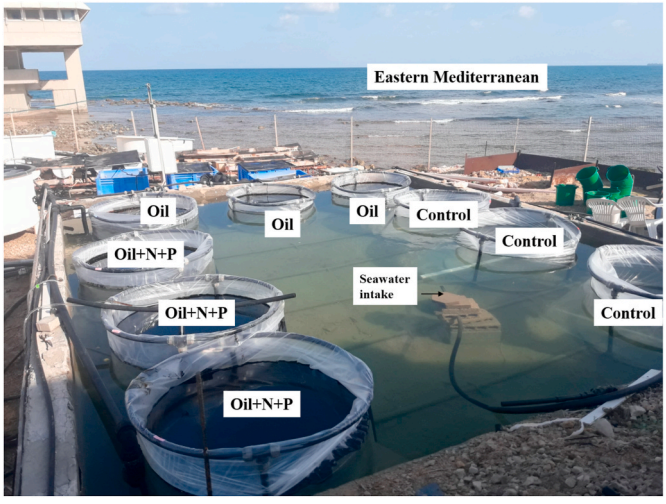

Fig. 1. The mesocosms experimental setup held in Tel-Shikmona (Haifa, southeastern Mediterranean coast) during winter 2017 and summer 2018. Triplicate mesocosms (1 m^3) were supplemented with crude-oil (500 nm thick), crude-oil+ nutrients, or remained unamended (control).

2.2. Inorganic nutrients

Seawater was collected from the mesocosms prior to the additions into acid-washed plastic scintillation vials and kept at −20 °C until analysis. Nitrate+nitrite (NO_2+NO_3), orthophosphate (PO_4) and silicic acid ($Si(OH)_4$) were determined using a segmented flow Seal Analytical AA-3 system (Kress et al., 2019). The limits of detection (two times the standard deviation of the blank) for NO_2+NO_3, PO_4 and $Si(OH)_4$ were 0.08 μM, 0.008 μM and 0.03 μM, respectively. No mid-point measurements were carried during the mesocosm experiments.

2.3. Chlorophyll.a (Chl.a)

Seawater samples (300 ml) were filtered onto 25 mm glass fiber filters (GF/F) using low vacuum (<150 mbar) and kept in amber glass scintillation vials at −20 °C until analysis. Chl.*a* was extracted from filters using 90% acetone overnight at 4 °C and determined by the non-acidification method (Welschmeyer, 1994) using a Turner Designs Trilogy® fluorometer at 436 nm excitation filter and 680 nm emission filter. Blank filters were also extracted in 90% acetone under the same conditions as the samples and their values were subtracted from the sample values.

2.4. Picophytoplankton and heterotrophic bacterial abundance

Synechococcus, *Prochlorococcus*, autotrophic picoeukaryotes and heterotrophic bacteria were enumerated by flow cytometry (Attune acoustic flow-cytometer). Seawater samples (1.8 ml) taken from the mesocosms were fixed with glutaraldehyde (Sigma G-7651, 0.02%), snap-frozen in liquid nitrogen, and kept at −80 °C until analysis. Prior to analysis, the samples were fast-thawed at 37 °C in a water bath. Taxon discrimination among autotrophic cells (*Synechococcus*, *Prochlorococcus* and autotrophic picoeukaryotes) was based on cell side-scatter, forward scatter, and orange (phycoerythrin, 585 nm) and red (Chl.*a*, 630 nm) fluorescence. For heterotrophic bacteria, subsamples (100 μl) were stained with SYTO9 (1:10^5 *v:v*) for 10 min in the dark, and these were enumerated based on cell side-scatter, forward scatter, and green fluorescence (530 nm).

2.5. Primary production (PP)

Seawater samples (50 ml) were collected in triplicates into transparent plastic tubes during the morning (~09:00), spiked with 5 μCi of $NaH^{14}CO_3$ (PerkinElmer, specific activity 1 mCi ml^{-1}) and incubated in the mesocosm bags for 4 h under ambient irradiance and temperature. Incubations were terminated by concentrating the particulate matter in each tube onto 25 mm GF/F filters and removing unbound bicarbonate by exposing the filters to 32% HCl vapors overnight. The total radioactivity was determined by a Packard Tri carb 2100 TR liquid scintillation counter (Steemann-Nielsen, 1952).

2.6. Bacterial production (BP)

Seawater samples (1.7 mL each) from each mesocosm were spiked with ~100 nmol ^{3}H-leucine L^{-1} (PerkinElmer, specific activity 130 Ci $mmol^{-1}$) and incubated in the dark for 4 h. The incubations were terminated with 100 μL of 100% trichloroacetic acid (TCA) and samples were processed following the micro-centrifugation technique (Smith et al., 1992). Disintegrations per minute (DPM) were counted using a

TRI-CARB 2100 TR liquid scintillation counter (Packard) following addition of 1 ml of scintillation cocktail (Ultima-Gold). TCA-killed samples (i.e., added at T0, before the incubation) were also prepared each day, and the DPM of these was subtracted from the sample DPM. A conversion factor of 1.5 kg C mol^{-1} per every mole of leucine incorporated was used (Simon and Azam, 1989).

2.7. DNA extraction, amplicon sequencing and data analysis

Seawater samples from each mesocosm (~4.5 L seawater) were filtered onto polycarbonate membrane filters (47 mm, 0.2 μm pore size, Merck Millipore, MA, USA) using a peristaltic pump. The membranes were stored at −20 °C until they could be processed. The membrane filters were cut in half and half of each filter was minced with a sterile scalpel and the DNA was extracted using the PowerSoil™ DNA Isolation Kit (QIAGEN, California, USA). The V4 region of the 16S rRNA gene in this material was amplified using the modified primer pair 515F-806R (Apprill et al., 2015; Parada et al., 2016) in combination with consensus sequence CS1/CS2 tags, using the following PCR amplification protocol: initial denaturation at 94 °C for 45 s, 30 cycles of denaturation (94 °C for 15 s), annealing (15 cycles at 50 °C and 15 cycles at 60 °C for 20 s) and extension (72 °C for 30 s). Library preparation and sequencing of 2 × 250 bp Illumina MiSeq reads was performed at HyLabs (Israel). Amplicon reads are available in NCBI Sequence Read Archive, BioProject PRJNA675315.

Demultiplexed paired end reads were analyzed using QIIME2 V2018.6 (Rideout et al., 2018). Reads were truncated based on quality plots, checked for chimeras, merged and grouped into amplicon sequence variants (ASVs) with DADA2 (Callahan et al., 2016), as implemented in QIIME2. A Naïve-Bayes classifier trained on the Silva 132 99% Operational Taxonomic Units (OTUs) from 515F/806R region of the 16S rRNA sequences was applied to the representative sequences to assign taxonomy. Representative sequences were aligned with MAFFT (Katoh and Standley, 2013), masked, and trees were generated using FastTree (Price et al., 2009), as implemented in QIIME2. Downstream statistical analyses and plotting were performed in R (R Core Development Team, 2013), using libraries phyloseq (McMurdie and Holmes, 2013), ampvis2 (Andersen et al., 2018) and ggplot2 (Wickham, 2009). Systematic changes across experimental conditions were estimated with DESeq2 (V1.26, Love et al., 2014). Permutation multivariate analysis of variance (PERMANOVA) and non-metric multidimensional scaling (NMDS) were based on Bray Curtis dissimilarities. Four samples (one from each of the following treatments: winter control T0, oil T2, T4 and summer control T4) were removed from the dataset due to insufficient reads yield. Organelle-derived sequence reads and singletons were removed from the dataset. Given that accurate estimation of alpha-diversity parameters with amplicon sequencing is currently under debate and development, we implemented only the beta-diversity analyses in this study.

2.8. Statistical analyses

All data processing was done using Excel XLSTAT software. A repeated measures analysis of variance (RM-ANOVA) was used to compare differences between the control, crude-oil and crude-oil+NO_3+PO_4 mesocosm treatments; the sampling days were defined as a repeated measures and treatments/seasons as the main factors ($p = 0.05$). Prior to the RM-ANOVA analyses, the normality and the heterogeneity of variances of the data were examined. Student's t-test was applied in order to compare the treatments from one another throughout the experimental duration (i.e., control vs. crude-oil, control vs. crude-oil+N+P and crude-oil vs. crude-oil+N+P).

3. Results and discussion

3.1. Initial water characteristics

There were distinct differences in water quality between the summer and winter samples taken from the SEMS coastal water (Table 1). During winter, surface water reached a low temperature of 17 °C and salinity was 38.6 psμ, whereas in the summer the temperature and salinity were 30 °C and 39.5 psμ, respectively. Concentrations of combined NO_2+NO_3, PO_4 and $Si(OH)_4$ in both seasons ranged between 0.24 and 0.39 μM, 0.04–0.05 μm, and 0.86–1.06 μM, respectively (Table 1). Chl.*a* levels were overall low (0.10–0.15 μg L^{-1}) and cyanobacteria (*Synechococcus* and *Prochlorococcus*) were the dominant autotrophic microbes (~90% of all pico-phytoplankton). *Synechococcus* abundances were similar in both seasons (~2000 cells ml^{-1}), while *Prochlorococcus* abundance was higher in summer than in winter by ~40% (985 vs. 707 cells ml^{-1}, respectively). Picoeukaryotes abundances were in the same order of magnitude as *Prochlorococcus*, but numbers were lower in the summer by ~30% than the winter values(524 vs. 752 cells ml^{-1}, respectively). Heterotrophic bacterial abundances were 1–2 orders of magnitude higher than those of cyanobacteria/picoeukaryotes, with a 5-fold higher abundance recorded in summer (~650,000 cells ml^{-1}) than in winter (~130,000 cells ml^{-1}). Primary production (PP) rates were ~2 fold higher in summer (0.84 ± 0.15 μg C L^{-1} h^{-1}) than in the winter (0.49 ± 0.06 μg C L^{-1} h^{-1}) and bacterial production (BP) rates ranged between 1.11 and 1.34 μg C L^{-1} h^{-1} in both seasons. This suggests a heterotroph-dominated system in both seasons (BP>PP), as has been shown previously for the SEMS waters (Rahav et al., 2013, 2018b; Raveh et al., 2015). Amplicon sequencing of the 16S rRNA genes illustrated large seasonal variation in taxonomic composition of microbial communities (Table S1, PERMANOVA, $p = 0.001$). At the beginning of the experiment (T0), summer communities were enriched in amplicon sequence variants (ASVs) that belonged to the Cryomorphaceae and NS5 marine group (Flavobacteriales), Puniceispirillales clade SAR116, and those classified as Rhodobacterales bacterium HIMB11, based on DESeq2 estimates (adjusted p-value<0.05). These summer communities are likely adapted to nutrient limitation. For example, Rhodobacterales bacterium HIMB11, which was very common during the summer, is able to synthesize glutamine lipids to substitute membrane glycerophospholipids as an adaptation to phosphorous limitation (Smith et al., 2019). During winter, taxa with higher nutrient requirements appeared, e.g. clade II of the ubiquitous marine alphaproteobacterium SAR11 and the ammonia-oxidizing archaea *Nitrosopumilus* (Salter et al., 2015; Stein, 2019). The abovementioned physicochemical and biological characteristics are in agreement with previous studies from the same study site, highlighting the oligotrophic status of this coastal environment (Raveh et al., 2015; Rahav et al., 2016, 2018b). However, since water temperature is a major factor governing microbial activity (Vázquez-Domínguez et al., 2007; Luna et al., 2012), we surmised that

Table 1
Summary of the initial chemical and biological characteristics of the SEMS water prior to the mesocosm experiments. Data shown are the averages ± standard deviations from 3 independent measurements.

Variable	Unit	Winter	Summer
Temperature	°C	16.9 ± 0.4	29.7 ± 1.1
Salinity	psu	38.6 ± 0.0	39.5 ± 0.2
NO_2+NO_3	μM	0.39 ± 0.14	0.24 ± 0.05
PO_4	μM	0.05 ± 0.02	0.04 ± 0.01
$Si(OH)_4$	μM	0.85 ± 0.11	1.06 ± 0.14
Chl.*a*	μg L^{-1}	0.10 ± 0.02	0.15 ± 0.03
Synechococcus	Cells ml^{-1}	2291 ± 290	1874 ± 314
Prochlorococcus	Cells ml^{-1}	707 ± 185	985 ± 116
Picoeukaryotes	Cells ml^{-1}	752 ± 120	524 ± 76
Bacterial abundance	Cells ml^{-1}	129,520 ± 10,674	652,547 ± 56,988
PP	μg C L^{-1} d^{-1}	0.48 ± 0.06	0.84 ± 0.15
BP	μg C L^{-1} d^{-1}	1.34 ± 0.11	1.11 ± 0.10

the response of the microbial community to anthropogenic oil input such as crude oil may differ between winter and summer.

3.2. Physiological responses of crude oil addition on bacterioplankton

Overall, crude-oil addition resulted in a decrease in autotroph biomass and activity during both the winter and summer experiments, whereas heterotrophic bacterial biomass and production rose (Figs. 2 and 3 and Tables 2 and 3). During winter, chl *a* levels decreased in the oil treatments by ~60% relative to the unamended controls 72 h post addition and thereafter (Fig. 2A). A stronger decline in algal biomass of 55–65% was recorded during the summer experiment 48–120 h post addition (Fig. 2B). The decrease in algal biomass was mostly explained by the marked decrease in *Synechococcus* abundances (maximal ~56%, Fig. 2C and D), and to a lesser extent to the drop in *Prochlorococcus* and pico/nano-eukaryotic algae that were more moderately affected (~10–20% reduction, Table 2). Heterotrophic bacterial abundance increased 48–72 h post crude-oil additions by 53–116% in both the winter and summer experiments (Fig. 2E and F). In terms of production, primary production rates dropped by up to ~95% in comparison to the controls following crude oil additions (Fig. 3A and B), whereas bacterial production increased by up to 165% (Fig. 3C and D).

The reduction in chl.*a* and PP may suggest that the added crude-oil induced phytoplankton mortality (El-Dib et al., 2010) or inhibited phytoplankton growth and photosynthesis (Hjorth et al., 2008; Fiala and Delille., 1999; Liu et al., 2006). Whatever the mechanism is, toxicity of crude-oil to phytoplankton is species-dependent and may cause cascading effects throughout the microbial food web (Ozhan and Bargu, 2014). For example, diatoms are more resistant than other phytoplankton such as cyanobacteria to crude-oil toxicity (González et al., 2009), possibly due to their lower surface-area-to-volume ratio (Echeveste et al., 2010). The degree to which the microbial community copes

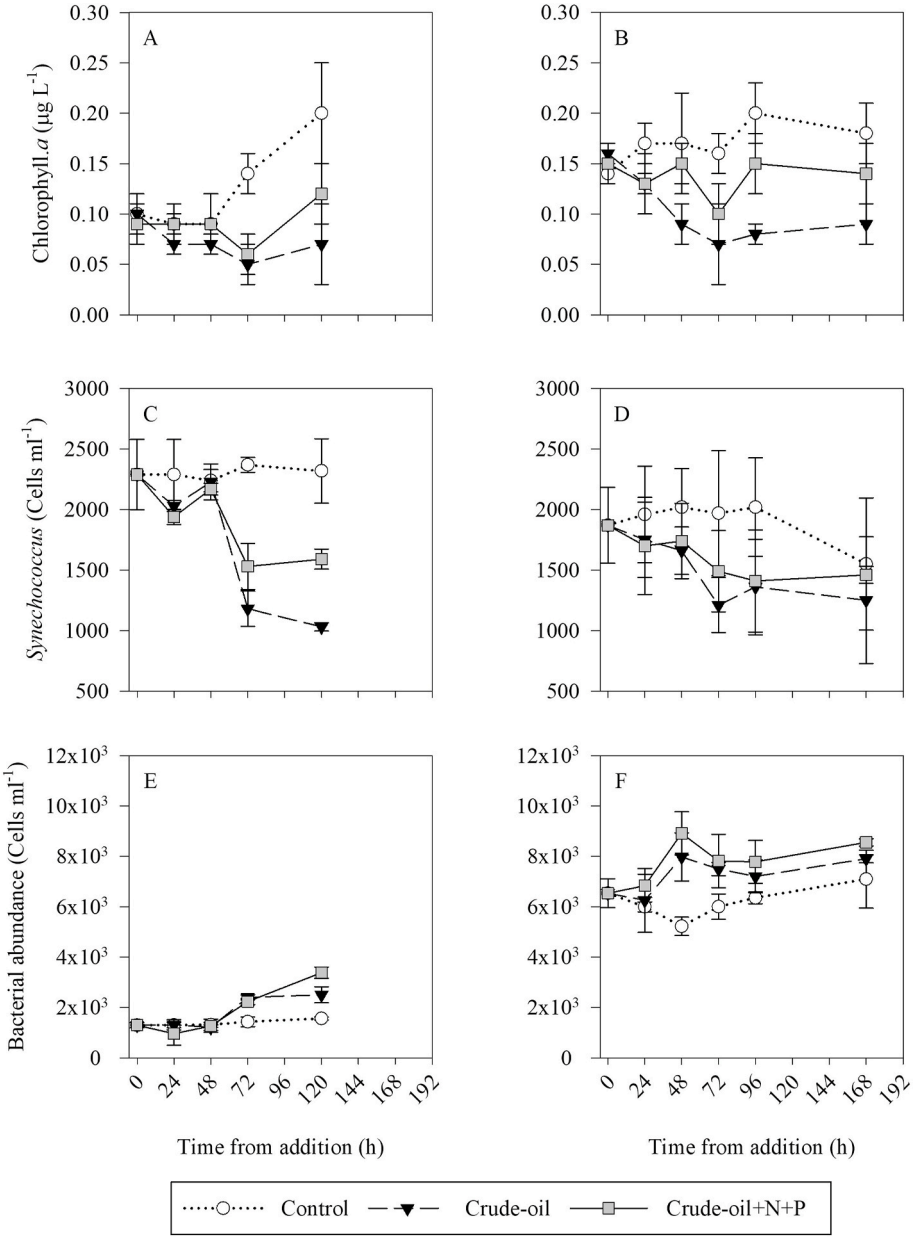

Fig. 2. Temporal variability in chlorophyll.*a* (A,B), *Synechococcus* (C,D) and heterotrophic bacteria (E,F) following crude-oil addition (black triangle), crude-oil-+nutrients addition (gray square) or unamended controls (white circle) during the winter (A,C,E) and summer (B,D,F) mesocosm experiments. Values presented are averages of 3 independent replicates and corresponding standard deviation.

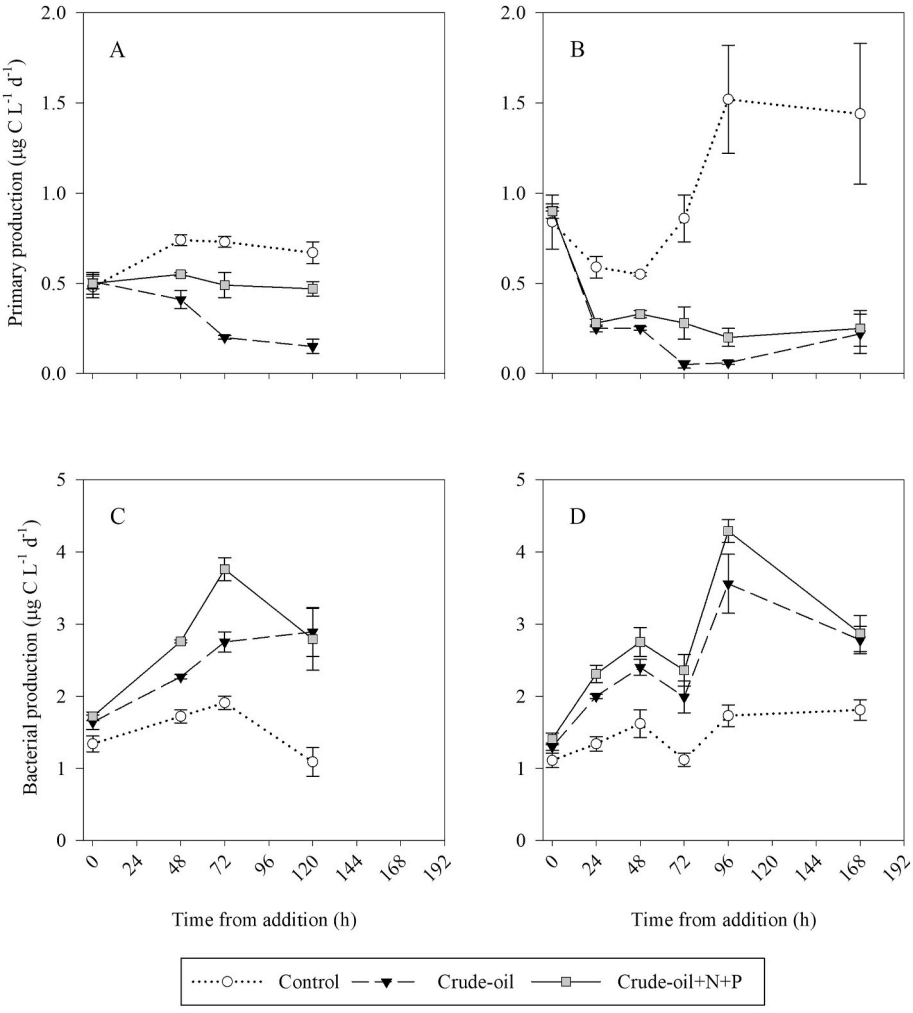

Fig. 3. Temporal variability in primary production (A,B) and bacterial production (C,D) following crude-oil addition (black triangle), crude-oil+nutrients addition (gray square) or unamended controls (white circle) during the winter (A,C) and summer (B,D) mesocosm experiments. Values presented are averages of 3 independent replicates and corresponding standard deviation.

Table 2
The maximal percent changes recorded relative to the unamended control mesocosms. In brackets the time where the changes were measured.

Variable	Crude-oil		Crude-oil+NO_3+PO_4	
	Winter	Summer	Winter	Summer
Chl.*a*	−65 (120 h)	−60 (96 h)	−52 (72 h)	−37 (72 h)
Synechococcus	−56 (120 h)	−39 (72 h)	−35 (72 h)	−30 (96 h)
Prochlorococcus	−63 (120 h)	−44 (48 h)	−37 (72 h)	−20 (72 h)
Picoeukaryotes	−24 (120 h)	−27 (96 h)	−17 (72 h)	−14 (96 h)
Bacterial abundance	+68 (72 h)	+53 (48 h)	+116 (120 h)	+71 (48 h)
PP	−78 (72 h)	−96 (96 h)	−33 (48 h)	−37 (96 h)
BP	+165 (72 h)	+106 (96 h)	+156 (120 h)	+148 (96 h)

Table 3
Results of a student t-test showing the differences between the mesocosm treatments throughout the experimental duration during the summer (4–120 h post addition) and winter (4–168 h post addition) experiments. Statistically significant differences ($p < 0.05$) are highlighted in bold.

Variable	Season	Control vs. Crude-oil	Control vs. Crude-oil+NO_3+PO_4	Crude-oil vs. Crude-oil+NO_3+PO_4
Chl.*a*	Summer	**0.02**	**0.02**	0.08
	Winter	**0.05**	0.07	0.06
Synechococcus	Summer	**0.01**	**0.02**	0.12
	Winter	**0.03**	**0.03**	0.15
Prochlorococcus	Summer	0.06	0.07	0.22
	Winter	**0.05**	0.06	0.10
Picoeukaryotes	Summer	0.10	0.08	0.23
	Winter	0.13	0.11	0.12
Bacterial abundance	Summer	0.06	**0.02**	0.20
	Winter	**0.01**	**0.01**	0.36
PP	Summer	**0.03**	**0.04**	**0.04**
	Winter	**0.04**	**0.03**	**0.04**
BP	Summer	**0.01**	**0.01**	**0.02**
	Winter	**0.03**	**0.01**	0.11

with oil contamination depends also on the microbe's uptake and excretion rates, as well as detoxification and repair mechanisms. Specifically, our results are in agreement with other studies that showed that crude-oil enrichment may drastically reduce cyanobacterial biomass and viability (Bacosa et al., 2015; Edwards et al., 2011). Cyanobacteria such as *Synechococcus* account for a substantial fraction of the marine primary production in the surface oceans (Flombaum et al., 2013), and their decrease may affect higher trophic levels as well (Almeda et al., 2014). Indeed, Brussard et al. (2016) showed that

oil-contaminated seawater provoked physiological dysfunctions in copepods on short timescales (1–3 days), despite their being motile with the ability to migrate to unpolluted waters. Unfortunately, due to the use of enclosed mesocosms in this study we could not monitor changes in zooplankton biomass, abundances and community composition. It is essential to carry out additional studies to understand the effects of crude-oil on zooplankton (along with bacteria and phytoplankton) in the SEMS water in order to fully evaluate how such anthropogenic pollutants affect other organisms within the microbial food-web.

In addition to the changes observed in microbial biomass and activity following crude-oil addition (Figs. 2 and 3), changes were also recorded in microbial diversity based on amplicon sequencing (Table S1, Fig. 3). While shifts in specific taxa were observed during both seasons, it was only in the summer experiment that we found a significant change in beta-diversity between the control and the oil-amended mesocosms 48 h post addition and onwards (PERMANOVA, $p = 0.001$). A major decrease in cyanobacterial relative abundance (mainly *Synechococcus*; Fig. 2C and D, Table 2) was observed 96 h after crude-oil amendments, reaching 3–5% in winter and less than 1% in summer (as compared to 13.4–27.2% in winter and 28.7–32.3% in summer at the beginning of the experiment, Fig. 4). The relative abundance of SAR11 decreased from 7.0 to 8.6% and 5.0–5.4% of sequences, at the beginning of the experiment in winter and summer, respectively, to 3.5% and 0.5% 48 h after oil addition (reaching 0.1% 96 h post addition in summer). These results confirm that crude-oil pollution results in the decline of the usually dominant marine microbial taxa, especially those inhabit oligotrophic regimes such as the SEMS (Dubinsky et al., 2016).

In winter, obligate hydrocarbonoclastic bacteria, primarily Altermonodales, Oceanospirillales, Rhodobacterales and Flavobacteriales lineages, became abundant 96 h post addition (Fig. 4). Differential analysis between the initial and 96 h post addition in the oil-amended mesocosm during winter revealed enrichment of *Glaciecola*, *Aestuariicella* and *Alteromonas* (Altermonodales), *Winogradskyella* (Flavobacteriales), *Shimia* and *Tropicibacter* (Rhodobacterales), (adjusted p-value<0.05). These taxa have the potential to degrade oil-derived hydrocarbons, while *Glaciecola* has the ability to produce biosurfactants (Dang et al., 2016; Gontikaki et al., 2018; Harwati et al., 2009; Krolicka et al., 2017; Lo et al., 2015; Peixoto et al., 2015). Comparison of summer oil-amended treatments at 96 h with controls, as well as with the time-zero point from the same treatment, revealed that potential hydrocarbon degraders *Altermonas* and *Winogradskyella* were enriched. It was interesting to note that *Alcanivorax* (Oceanospirillales), the metabolizer of oil-derived alkanes (Gutierrez et al., 2013; Yakimov et al., 2007), and *Caulobacterales* spp., which may degrade alkanesulfonates (Abbasian et al., 2016) were only detected in the summer oil-amended mesocosms. Moreover, oil additions in summer mesocosms resulted in enrichment of bacterial lineages that may be involved in the secondary degradation of oil-derived biomass, such as Planctomycetes (Arnosti et al., 2015).

The immediate response observed in bacterial abundance, production and diversity suggests that the SEMS water has a constant-basal level of microbial oleophiles. This is in agreement with an observation from the offshore deep waters of the SEMS where oil-degrading bacteria such as *Alcanivorax* spp. were found near the nutricline and below it (Keuter et al., 2015). Thus, once the surface waters are enriched with hydrocarbons, these local oil bio-degraders become abundant, utilizing carbon from the crude-oil and NO_3+PO_4 from the surface waters. This response creates a microbial cascade, leading to reduced phytoplankton biomass and production, likely due to N or N&P co-limitation in nearshore microbial populations (Kress et al., 2005; Rahav et al., 2018a; Raveh et al., 2019). It should be noted that N and P levels were not quantified throughout the experiment and therefore we could not substantiate or refute this hypothesis. Nevertheless, previous studies from the SEMS showed that heterotrophic bacteria may outcompete phytoplankton for PO_4 (Thingstad et al., 2005) or dissolved organic P (Sisma-Ventura and Rahav, 2019). We therefore hypothesized that the addition of NO_3+PO_4 to crude-oil will simulate the growth and activity of hydrocarbon bio-degraders, thereby minimizing the deleterious

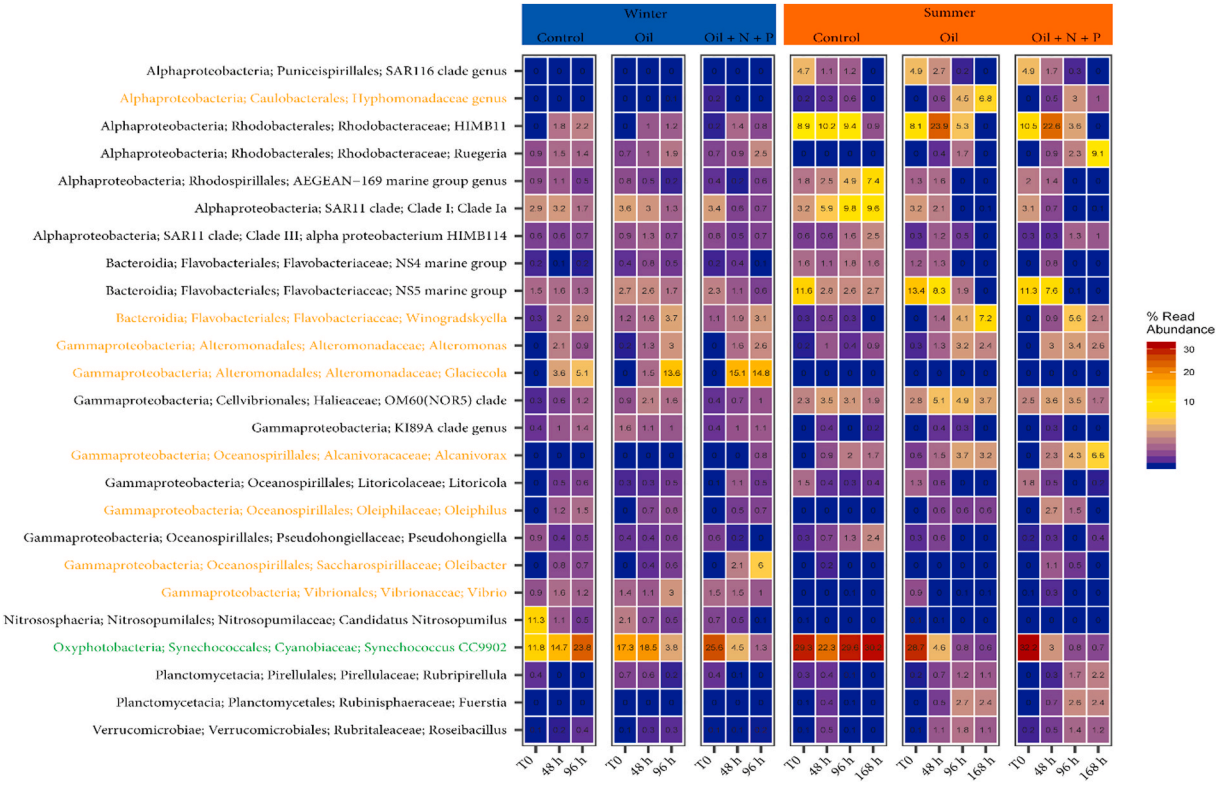

Fig. 4. A heat map showing the most abundant bacterial families derived from 16S rRNA OTUs analyses during winter (blue header) and summer (orange header) mesocosm experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

effects of oil pollution on the already nutrient-limited phytoplankton.

3.3. The response of microbial communities to amendments of NO_3+PO_4 and crude oil

The fertilization of oil-enriched seawater with nutrients leads to reduced inhibitory effects on phytoplankton when compared to the nonfertilized equivalents (Figs. 2,3). Thus, chl.*a* decreased in the mesocosms treated with crude-oil+NO_3+PO_4 by 37–52%, which is lower than that recorded following crude-oil amendments alone (>60%, Fig. 2A and B, Table 2). Correspondingly, *Synechococcus* abundance and PP decreased moderately (maximal change ~30–35%) compared to the marked decline observed following crude-oil alone (39–56% and 78–96%, respectively, Table 2, Fig. 2C and D, Fig. 3A and B). On the other hand, heterotrophic bacterial abundance (71–116%) and bacterial production (148–156%) rapidly increased, similarly to that observed following crude-oil alone (Fig. 2E and F, Fig. 3C and D, Table 2). These results suggest that the added NO_3+PO_4 reduced the stress caused by nutrient deficiency to phytoplankton (Rahav et al., 2016, 2018a; Raveh et al., 2019) and thus helped in their succession, post-oil-exposure.

Another possibility is that the added nutrients enhanced oil degradation by heterotrophic bacteria, which can indirectly be observed by the enhanced BP. Hydrocarbon degraders may outcompete phytoplankton for surface water's NO_3+PO_4, as well as for (Leahy and Colwell, 1990), resulting in lower phytoplankton growth and activity, as observed in our experiments (Figs. 2 and 3). Contrary, addition of NO_3+PO_4 may alleviate nutrient limitation and thus benefit phytoplankton succession (Ozhan and Bargu, 2014). Increase in phytoplankton biomass may further reduce the toxic impact of hydrocarbons since the exposure to hydrocarbons becomes diluted, resulting in lower concentrations of toxic compounds per individual organism under nutrient enriched conditions (Skei et al., 2000; Ozhan and Bargu, 2014).

In accordance with the physiological and biomass changes, significant alterations in microbial diversity were recorded following crude-oil+NO_3+PO_4 compared to the crude-oil alone (Fig. 4). In winter, but not in summer, we observed significant differences in beta diversity of microbial communities between crude-oil mesocosms amended with NO_3+PO_4 and those without the nutrient additions (PERMANOVA, $p = 0.037$ and $p = 0.654$, respectively). In winter, a large suite of obligate oil degraders were enriched following nutrient amendments, such as *Oleibacter*, *Oleispira*, *Neptuniibacter*, *Pseudoalteromonas*, *Thalassotalea*, *Tropicibacter*, *Pseudophaeobacter* and *Sulfitobacter* (Dombrowski et al., 2016; Giebel et al., 2016; Harwati et al., 2009; Krolicka et al., 2017; Teramoto et al., 2011; Thiele et al., 2017; Wawrik et al., 2012; Yakimov et al., 2007), as well as a few ASVs classified as KI89A clade spp. and Kiloniellaceae spp., which are not known to degrade oil hydrocarbons (enrichment calculated with DESeq2 for both 48 and 96 h timepoints together, adjusted p-value cutoff = 0.05). The reciprocal analysis revealed enrichment in SAR11 clade ASVs when no nutrients were added to mesocosms with oil. In summer, no significant enrichments were observed in nutrient-amended mesocosms when all three time-points were tested together. However, after removal of the 48 h time point, in which the communities were highly similar between the crude oil only and the nutrient amended+crude oil treatments, comparative analysis revealed enrichment of ASVs classified as potential Rhodobacteraceae degraders of oil *Ruegeria* and *Thalassobius* (Bacosa et al., 2015; Giebel et al., 2016), as well as a methylotroph *Methylophilus*, which was previously found in oil-polluted environments (Dombrowski et al., 2016). A different *Thalassobius* ASV, as well as those classified as BD1-7 clade (Spongiibacteraceae) and NS5 marine group (Flavobacteriaceae) were enriched in the cruise-oiltreatments. Hence, nutrient availability may affect not only the activity but also the diversity of oil-degrading microbial communities.

4. Conclusions

Crude-oil pollution in marine systems, and especially in landlocked seas such as the Mediterranean Sea, is a great ecological concern, in particular in light of the rapid growth in exploration and exploitation of marine petroleum reserves and oil transport. The diverse intrinsic hydrocarbon-degrading microbiota found here, regardless of any addition or manipulation (control mesocosms), suggests that the SEMS coastal water are chronically exposed to some level of crude-oil pollution.

Our results demonstrate that crude-oil contamination in the coastal SEMS has deleterious effects on phytoplankton, in particular *Synechococcus*, and on primary production rates. These deleterious effects are stronger under nutrient-impoverished conditions which are typical to this system. We have found that the addition of limiting nutrients (*e.g.,* NO_3 and PO_4) reduced the inhibitory effects of crude-oil contamination on phytoplankton. Thus, fluctuations in nutrient regimes within the coastal SEMS may play a major role in determining the phytoplankton response to crude-oil exposure. Determining the recovery rates of dominant phytoplankton species and studying the potential alterations and physiological adaptation of phytoplankton (as well as zooplankton) to crude-oil spills in the SEMS coastal water is needed to achieve better science-based management of oil spills, as well as better prediction and mitigation of long-term effects. Moreover, the contamination area of crude-oil following spills can penetrate subsurface and spread hundreds of meters away from the source. This may affect different phytoplanktonic communities that inhabit different depths and therefore may require different responses.

One of the commonly used strategies to cope with oil spill pollution is the addition of dispersants as a mean to increase the oil's bioavailability for bacteria in the water column (Dave and Ghaly, 2011). Our results suggest that in the event of an oil spill in the SEMS coastal water, biodegradation rates are likely to be slow, even after dispersants have been used, due to the extreme nutrient starvation of microbes in this system, and the typical slow growth rates of marine bacteria in LNLC regimes (usually $>1\ d^{-1}$, Kirchman, 2012). Under these circumstances, the use of dispersants may not be effective and will probably be deleterious. Thus, controlled local eutrophication by application of fertilizers to the polluted area should be considered as part of the remediation program after a major oil spill in the SEMS, as was done after the Exxon Valdez oil spill (Dave and Ghaly, 2011). We therefore stress that SEMS-specific oil-spill remediation techniques should be tested and/or developed. The dynamics in phytoplankton/bacteria in response to oil should be studied further, in line with UNEP's Integrated Monitoring and Assessment Program for the Mediterranean Sea (IMAP) as part of the Barcelona Convention Mediterranean Action Plan. This is essential for the establishment of a toolset to detect small-scale oil pollution events and to determine the effects of future large-scale ones on the marine environment.

CRediT authorship contribution statement

Yael Shai: Conceptualization, Formal analysis, Data curation, Writing - original draft, Conceived and designed the experiment, Performed the experiment. **Maxim Rubin-Blum:** Formal analysis, Data curation, Writing - original draft, Contributed reagents/materials/analysis tools. **Dror L. Angel:** Conceptualization, Writing - original draft, Conceived and designed the experiment. **Guy Sisma-Ventura:** Formal analysis, Data curation. **Dror Zurel:** Formal analysis, Data curation, Writing - original draft. **Peleg Astrahan:** Formal analysis, Data curation. **Eyal Rahav:** Conceptualization, Formal analysis, Data curation, Writing - original draft, Conceived and designed the experiment, Performed the experiment, Contributed reagents/materials/analysis tools.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2020.107102.

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