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Dark inorganic carbon fixation contributes to bacterial organic carbon demand in the oligotrophic Southeastern Mediterranean Sea

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Abstract

Settling organic matter derived from photosynthesis at the illuminated layers is often not sufficient to meet the energy demands of microbes in the dark ocean. This 'mismatch' is especially notable in the warm and oligotrophic Mediterranean Sea where the annual photosynthesis is one of the lowest in the world's oceans yet its aphotic zone is considered a hotspot for microbial activity and biomass. Here, we investigated the role of photic and aphotic dark inorganic carbon fixation rates (DCF) in supporting

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bacterial carbon demand at the offshore south-eastern Mediterranean Sea during the mixed and stratified periods. Our results demonstrate that DCF rates are measurable throughout the water column (0-1750 m) and are on the same order of magnitude as photosynthesis (34 vs. 45 g C m^{-2} y^{-1}, respectively). Using a carbon mass balance that considers photosynthesis, DCF and bacterial production (and hence respiration) we show that chemoautotrophy provides ~35% of the 'missing carbon' supply needed for microbial growth and activity in the aphotic layer, while other sources of dissolved organic carbon remain to be elucidated. These findings underscore the need for further research into the factors affecting DCF, its role in global carbon budgets, and its potential to enhance atmospheric inorganic carbon sequestration.

1 Introduction

The oceans aphotic layers contain the world's largest reservoir of dissolved inorganic carbon (DIC) pool (Burd et al., 2010; Reinthaler et al., 2010), and harbor ~65% of all prokaryotes (Whitman et al., 1998). Aphotic prokaryotes typically rely on utilization of organic matter (and carbon) exported from the euphotic zone through photosynthesis to sustain their growth and accumulate biomass (del Giorgio and Duarte, 2002). Current estimates reveal, however, a discrepancy between the supply of particulate organic carbon from photosynthesis and the bacterial organic carbon demand (BCD) in the aphotic zones (Ducklow, 2000; Karl et al., 1988; Smith and Azam, 1992). This mismatch suggests that there are other source/s of carbon that are being utilized by aphotic microorganisms (Baltar et al., 2009; Herndl and Reinthaler, 2013). One such source, that has been far less investigated, involves the fixation of DIC by chemo-autotrophic microbes and its assimilation into new biomass. This could subsequently provide bioavailable DOC to other microbial populations at depth.

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DIC uptake by heterotrophic bacterioplankton is generally attributed to anaplerotic reactions (Dijkhuizen and Harder, 1984; Erb, 2011), but other microorganisms such as nitrifying bacteria can also fix DIC (Alonso-Sáez et al., 2010). Genomic studies on deep-sea microbial communities identified several genes and metabolic pathways that enable some microbes to thrive as chemoautotrophs on inorganic substrates (Berg et al., 2007; Hallam et al., 2006). Measurements of CO_2 fixation by chemoautotrophs and heterotrophic bacterioplankton are scarce, yet substantial dark DIC fixation (DCF) rates have been reported in various oceanic settings and water masses (Swan et al. 2011; Zhou et al. 2017; La Cono et al. 2018; Alothman et al. 2023) and maybe more common than previously thought (Hansman et al., 2009; Herndl et al., 2005).

The deep waters of the southeast Mediterranean Sea are characterized by higher concentrations of inorganic nutrients compared to the photic zone (e.g., ~6 µmol NO_3+NO_2 kg^{-1} and ~0.2 PO_4 µmol kg^{-1} (Ben-Ezra et al., 2021; Sisma-Ventura et al., 2021) and low bioavailable dissolved organic carbon (Martínez-Pérez et al., 2017; Santinelli, 2015; Santinelli et al., 2010). Despite these characteristics, the southeast Mediterranean Sea's aphotic waters are considered a hotspot for bacterial activity compared to other oceanic regimes at similar depths (Luna et al., 2012; Rahav et al., 2019). Nutrient addition bioassays and water mixing simulations suggest that aphotic prokaryotes are primarily carbon-limited (Hazan et al., 2018; Rahav et al., 2019).

Here, we report on both photic and aphotic DCF and heterotrophic bacterial production rates from 6 cruises held between 2021-2023 at the offshore southeastern Mediterranean (bottom depth 1500-1750 m) during the mixed (winter) and stratified (summer) periods. Our results demonstrate that DCF rates cannot be negleted (contrary to past conventions, Nielsen 1952) and within the same order of magnitude as

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photosynthesis or heterotrophic bacterial production (BP). We also show that DCF substantially contributes to bacterial carbon demand (BCD), therefore providing, some of the 'missing carbon' supply needed for microbial growth and activity in the aphotic layer of the southeast Mediterranean Sea.

2 Material and methods

2.2 Sample collection - Seawater was collected during six seasonal cruises in the Levantine Basin, southeast Mediterranean Sea, on-board the R/V *Bat-Galim* between 2021-2023. Two 'deep' stations were sampled in each cruise; one located at the edge of the continental shelf (H05 33.00 Lat, 34.50 Lon, bottom depth ~1500 m, 50 Km from the coast) and the other at the edge of Israel's exclusive economic zone (H06 33.15 Lat, 34.16 Lon, bottom depth 1750 m, 90 Km from shoreline). Seawater was sampled at discrete depths throughout the water column, from the surface (~0.5 m) to the bottom (1500-1750 m) using Niskin bottles. Sampling depths were chosen in real-time based on reads of Conductivity Temperature Depth (CTD) (Seabird 19 Plus), chlorophyll fluorescence (Turner designs, Cyclops-7) and PAR (Sea Bird). Measurements included DIC ($NaH^{14}CO_3$) uptake under ambient light (hereafter 'light primary productivity, LPP) or under full dark conditions (DCF), bacterial productivity (BP) and nutrient quantification.

Nitrite and ammonium concentrations – Samples for nitrite (NO_2^-) and ammonium (NH_4^+) concentrations were collected only in March and August 2023 cruises. The samples were pre-filtered (0.45 µm), placed in acid-washed plastic vials, and were kept frozen at -20 °C until analysis. Nutrients were measured with a Seal Analytical AA-3 system. The limits of detection for NO_2^- and NH_4^+ were 0.06 µM and 0.09 µM, respectively.

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2.3 LPP and DCF - Seawater was collected in triplicates into 45-250 ml bottles and spiked with $NaH^{14}CO_3$ (Perkin Elmer, specific activity 56 mCi mmol^{-1}). The bottles were incubated for 24 h under ambient light for 'total primary production or under full dark for DCF estimates (Nielsen, 1952). At the conclusion of the incubation, the spiked seawater was filtered onto GF/F filters using low vacuum pressure (<50 mmHg). Next, the excess ^{14}C-bicarbonate was removed by fuming with 50 µl of 37% hydrochloric acid overnight. Finally, 5 ml scintillation cocktail (ULTIMA-GOLD) was added, and the disintegrations per minute (DPM) from the particulate matter concentrated on the filters were counted using a TRI-CARB 4810 TR (Packard) liquid scintillation counter. Blank seawater spiked with $NaH^{14}CO_3$ was filtered immediately without incubation and the reads were subtracted from the sample's DPM. The blank DPM reads were usually negligible (<5% of the sample's DPM). Aliquots (50 µl) from random spiked samples were placed onto new GFF filters, added with 50 µl ethanolamine and scintillation liquid, and counted immediately without incubation to account for the 'added activity' of the radiolabeled working solution used. LPP was calculated as the difference between the DPM retrieved from the samples incubated under ambient light ('total primary production) and the 'dark' bottles. Dark or light dissolved inorganic carbon fixation was calculated based on the Bermuda Atlantic Time-series Study (BATS) protocol (https://bios.asu.edu/bats/bats-data). More details can be found in Reich et al. (2024).

2.3 Bacterial production- Samples (1.7 ml) were incubated in the dark with 100 nmol ^{3}H-leucine L^{-1} (Perkin Elmer, specific activity 123 Ci mmol^{-1}) for 4-6 h under ambient temperature (Simon et al., 1990). The incubations were terminated with 100 µl of trichloroacetic acid (100%), were processed as described by Smith and Azam (1992), and counted using a TRI-CARB 4810 TR (Packard) liquid scintillation counter. Killed

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control samples containing ^{3}H-leucine L^{-1} and trichloroacetic acid (without incubation) were also measured and this control sample's DPMs were subtracted from the sample's reads. A conversion factor of 3 kg C mol^{-1} per mole leucine incorporated was used, assuming an isotopic dilution of 2.0 (Simon and Azam 1989).

2.4 Molecular analyses and statistics - DNA was extracted from water samples with the PowerWater kit (Qiagen, USA), using the FastPrep-24™ Classic (MP Biomedicals, USA) bead-beating to disrupt the cells (2 cycles at 5.5 m sec-1, with a 5 min interval). The V4 region (~ 300 bp) of the 16S rRNA gene was amplified from the DNA (~50 ng) using the 515Fc/806Rc primers amended with relevant tags(Apprill et al., 2015; Parada et al., 2016). PCR conditions were as follows: initial denaturation at 94 °C for 45 s, 30 cycles of denaturation (94 °C for 15 sec), annealing (15 cycles at 50 °C and 15 cycles at 60 °C for 20 sec) and extension (72 °C for 30 s). Two annealing temperatures were used to account for the melting temperature of both forward (58.5-65.5 °C), and reverse (46.9-54.5 °C), primers.

Demultiplexed paired-end reads were processed in QIIME2 V2022.2 environment (Bolyen et al., 2019). 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. The amplicons were classified with Scikit-Learn classifier that was trained on Silva database v138 (16S rRNA, (Glöckner et al., 2017). Mitochondrial and chloroplast sequences were removed from the 16S rRNA amplicon dataset. Downstream analyses were performed in R v4.1.1 (R Core Team, 2021), using packages Phyloseq (McMurdie and Holmes, 2013) and Ampvis2 (Andersen et al., 2018). Indicator species analyses were performed using Indic species package v1.7.9 (De Ca´ceres et al., 2009). Amplicon reads were deposited to the NCBI SRA archive under project number PRJNA1215023.

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3 Results and discussion

LPP was restricted to the photic layer with highest rates usually measured at the surface (~0.5 m) that gradually decreased to reach minimum rates at the bottom of the photic layer (~180 m) (Fig 1A). Relatively low LPP values were measured during the stratified summer (~0.1-0.8 μg C L^{-1} d^{-1}), whereas higher rates were measured during the winter mixing period (~0.2-7.4 μg C L^{-1} d^{-1}) (Fig 1A). This resulted in ~10-fold higher integrated rates measured during the mixed compared to those measured during the stratified period (Table 1), in accordance with studies from the area (Psarra et al., 2005; Reich et al., 2022; Sisma-Ventura et al., 2022b). In contrast with LPP, DCF was not restricted to the photic layer and ranged from 0 to ~0.4 μg C L^{-1} d^{-1} throughout the water column (Fig 1B, Fig 2A), without significant differences in the absolute rates between the photic and aphotic zones (t-test, p>0.05, Fig 2B). The integrated photic DCF was typically lower, yet rates were of the same order of magnitude, then others previously reported in the central and western Mediterranean Sea (La Cono et al., 2018). The aphotic DCF rates were ~3.5 fold higher during the mixed than during the stratified period (Table 1, Fig 2A).

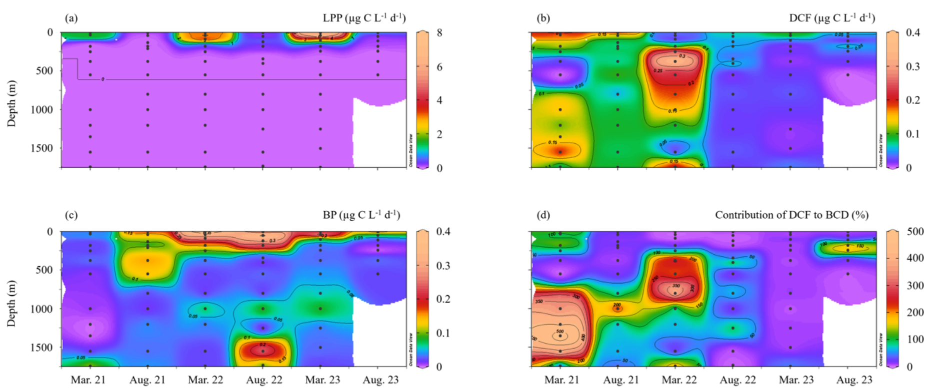

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Figure 1: Spatial and temporal variability in rates of LPP (a), DCF (b), BP (c) and the contribution of DCF to bacterial carbon demand (BCD) (d) at the offshore SE Mediterranean Sea (Lat. 33.15 N, Lon. 34.16 E) between 2021-2023. BCD was calculated assuming a bacterial gross efficiency of 0.20 (Gasol et al., 1998).

Table 1: Rates and contribution of DCF to metabolic processes in the photic (0-180) and aphotic (>180 m) depths of the pelagic southeast Mediterranean Sea BDL – Below detection limit.

Variable	Season	Photic (0-180 m)	Aphotic (180-1750 m)
LPP (mg C m^{-2} d^{-1})	Mixed	158-649	BDL
	Stratified	4-69	BDL
DCF (mg C m^{-2} d^{-1})	Mixed	6-27	17-342
	Stratified	7-19	8-127
BP (mg C m^{-2} d^{-1})	Mixed	6-58	12-65
	Stratified	9-55	7-123
DCF contribution to BCD (%) *	Mixed	23-221	49-594
	Stratified	8-31	8-42
DCF contribution to total PP (%)	Mixed	1-15	---
	Stratified	12-81	---

* Assuming bacterial gross efficiency of 0.2 (Gasol et al., 1998) and that the available DOC for bacteria is 20% of the total primary productivity at the photic layer (Teira et al., 2003).

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The higher aphotic DCF may be related to more bioavailable carbon that is transported from the photic layer as marine snow and supplies organic carbon to heterotrophic activity (mostly during winter when LPP is higher). However, given the oligotrophic nature of the southeast Mediterranean Sea, including during the winter (Berman-Frank and Rahav, 2012; Reich et al., 2022), most of the organic carbon (both particulate and dissolved originating from LPP is recycled within the photic layer and only a small fraction fluxes down to the aphotic zone) as has been recorded in sediment traps (Alkalay et al., 2024).

Another possible mechanism that may, potentially, explain higher DCF rates during the winter versus the summer is anaplerosis. The extent of anaplerotic reactions is primarily driven by the availability of labile organic carbon to heterotrophs (Dijkhuizen and Harder 1984). Therefore, assuming anaplerosis drives DCF, we expect it will be positively coupled to BP. Yet, our results do not support the likelihood of significant anaplerosis reactions, mainly because, the spatiotemporal distribution of aphotic BP (Fig 1C) differs considerably from that of the DCF (Fig 1B) and does not correlate with it (Fig 3A). In fact, BP seems to be coupled with LPP at the photic layer reaching ~0.4 $\mu g\ C\ L^{-1}\ d^{-1}$ (not shown). Except for some sporadic measurements, aphotic BP rates were usually homogeneous and typically <0.1 $\mu g\ C\ L^{-1}\ d^{-1}$ (Fig 1C). Further, the highest integrated aphotic BP was measured during the summers of 2021 and 2022 and not during the winter cruises when generally higher DCF was recorded (Table 1, Fig 2A).

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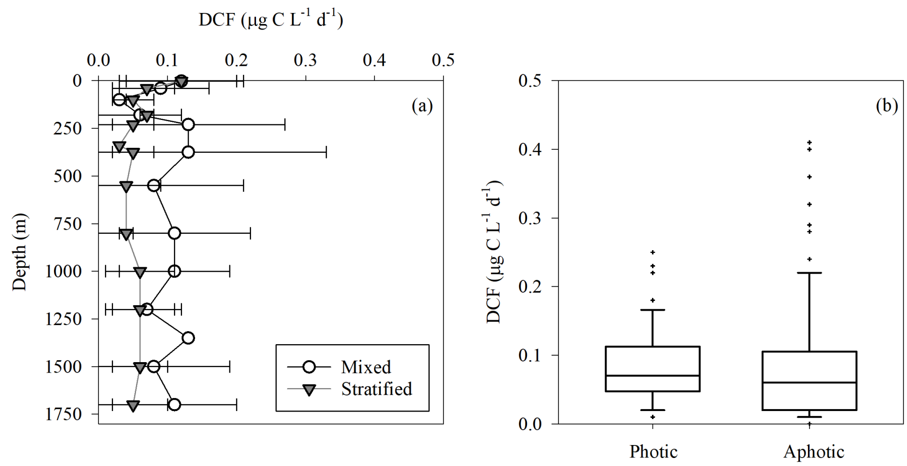

Figure 2: Dark carbon fixation rates in the southeastern Mediterranean Sea. Averaged vertical distribution of DCF in the offshore southeast Mediterranean Sea during the mixed (white) and stratified (gray) periods (a), and a box plot showing the DCF rates at the photic (0-180 m) and aphotic (>180 m) water depths (b).

Despite the lack of a clear positive relationship between DCF and BP, DCF may contribute to bacterial carbon demand (BCD) at the aphotic zone. Thus, we use a literary standard, a bacterial growth efficiency of 0.20 (Doval et al., 2001) to calculate BR and BCD. This resulted in bacterial respiration (BR) ranging from 29-494 mg C m^{-2} d^{-1} (average 209±172 mg C m^{-2} d^{-1}), and the concurrent BCD ranges from 36 to 648 mg C m^{-2} d^{-1} (average 262±121 mg C m^{-2} d^{-1}). Under these circumstances, exudation of DOC from primary productivity at the photic layer estimated as 20% of the rates (Teira et al., 2003) and equals ~1-130 mg C m^{-2} d^{-1}. This new DOC, the originated form the photic zone cannot support the aphotic BCD in our system in all of our observations. However, if we consider the contribution of DOC produced by aphotic DCF, part of the missing carbon can be 'bridged'. Thus, when considering aphotic DCF in addition

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to the sequestered DOC from the photic layer, the 'abnormally high' aphotic BCD could be explained in full (≥100%) in ~35% of the observations (Fig 1D). In the other 65% of the observations the missing carbon sources needed to support the aphotic BCD remains an enigma. Evidences suggest that dissolved methane may be more abundant in oxygenated environments than previously thought (Grossart et al., 2011). Methane can potentially be one of the missing energy sources for marine microbes and support high BCD (Brankovits et al., 2017) as observed at the aphotic southeast Mediterranean Sea (Fig 1D). In agreement, methanotrophs were found in aphotic cold seeps at the southeast Mediterranean Sea (Sisma-Ventura et al., 2022a), as well as across the aphotic water column in our samples (see discussion below).

Interannual variability in DCF, but not in LPP or BP, was observed with higher rates recorded in March 2021-March 2022 and lower rates observed in August 2022-August 2023 (Fig 1B). The drivers of this variability are unknown and may be related to differences in nutrients availability that fuels chemoautotrophic microbes of the aphotic layer between these two periods. While the absolute concentrations of PO_4^{3+} or NO_3^- $+NO_2^-$ in the deep aphotic water were similar overall between periods (not shown) and, therefore, not likely to affect the DCF rates, organic nutrients that were not considered here may limit chemoautotrophy. In support, recent studies from the northern Red Sea and South China Sea showed that DCF at the surface water is limited by labile organic nutrients such as phosphonates and even carbon-rich molecules (Reich et al., 2024; Zhou et al., 2017). Aphotic free-living chemoautotrophs are likely to encounter an increasingly refractory pool of dissolved organic matter for metabolism that may result in lower DCF rates, as shown exported through the water column in Bar-Zeev et al. 2015. Particle-attached chemoautotrophs may have access to higher concentrations of organic substrates, and therefore we surmise they have a preferential particle-attached

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lifestyle in the deep ocean. The patchy nature of particulate matter sinking (Alkalay et al., 2024) and aggregate concentrations (Bar-Zeev et al., 2012) in the deep southeast Mediterranean Sea could also potentially explain the interannual variability in DCF between periods. Understanding how chemoautotrophs transform labile dissolved organic matter into refractory dissolved organic matter, a process known as the 'microbial carbon pump' (Herndl and Reinthaler, 2013), is crucial as it influences the efficiency of the biological pump (Jiao et al., 2010).

Oxidizing reduced inorganic compounds as electron donors (e.g., NO_2^- or NH_4^+) may provide chemoautotrophic prokaryotes sufficient energy to fix DIC (Hügler and Sievert, 2011). We therefore measured (March and August 2023 cruises) the vertical distribution of NO_2^- and NH_4^+ and examined if these chemical species are coupled/uncoupled with DCF at the aphotic zone. Indeed, our results show a negative-linear relationship between DCF and NO_2^- (Fig 3B) and NH_4^+ (Fig 3C), suggesting nitrification is potentially an important metabolic pathway yielding energy to fix DIC in the aphotic zone. In agreement, both ammonia and nitrite oxidizers were found in the aphotic zone of all cruises (discussion below), further highlighting their potential role as contributors to DCF in southeast Mediterranean Sea. Nitrification measurements along with metagenomic tools, DCF (and BP) in aphotic water should be done in future dedicated studies to better refute or reinforce that oxidation of NO_2^- or NH_4^+ may provide chemoautotrophic prokaryotes the energy to fix DIC.

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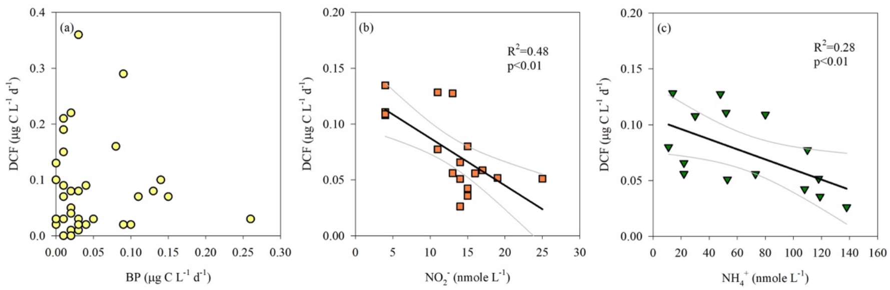

Figure 3: Possible relationships examined via linear correlations between aphotic DCF and BP (a), NO_2^- (b) and NH_4^+ (c). Note that NO_2^- and NH_4^+ was measured only during the 2023 cruises. The 95% confidence interval is shown in gray.

Analyses of 16S rRNA gene amplicons suggest that diverse bacteria and archaea may drive DCF in the aphotic southeast Mediterranean Sea (Fig 4A). Microbes found in our collected genetic material primarily include the order Nitrosopumilales ammonia-oxidizing archaea, which become dominant below DCM (up to ~30% read abundance near the bottom), corresponding to previous estimates based on *in-situ* fluorescent hybridization (De Corte et al., 2009). Nitrite-oxidizing Nitrospirales comprised ~1% read abundance at depths below 300 m. Among these lineages, analyses of indicator species identified seasonal variation in abundance of the orders Nitrosopumilales and Nitrososphaeria that were more prominent in the stratified period than in wintertime (p-value<0.05), while the deep-sea community in general exhibited only mild seasonal changes (Fig 4B). These ammonia oxidizers may thus drive ammonia depletion during summertime at the southeast Mediterranean Sea.

Additionally, we identified consistent occurrence of UBA10353 (Arenicellales, including the UBA868 group) and SAR324 gammaproteobacterial groups at depths below the deep chlorophyll maxima (Fig 4A). Members from these ubiquitous clades are mixotrophs that can fix inorganic carbon conserving energy from sulfur oxidation

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(Baltar et al., 2022; Jaffe et al., 2024; Swan et al., 2011). We did not find the SUP05/Arctic96BD-19 gammaproteobacterial sulfur oxidizers that occur in productive dark oxygenated waters (Swan et al., 2011). We note that Methylococcales methane oxidizers were typical in the near-bottom water layer, as found previously in the southeast Mediterranean Sea basin (Sisma-Ventura et al., 2022a; Techtmann et al., 2015), suggesting methylotrophy as a potential mechanism of 1-carbon molecule acquisition in the dark southeast Mediterranean Sea.

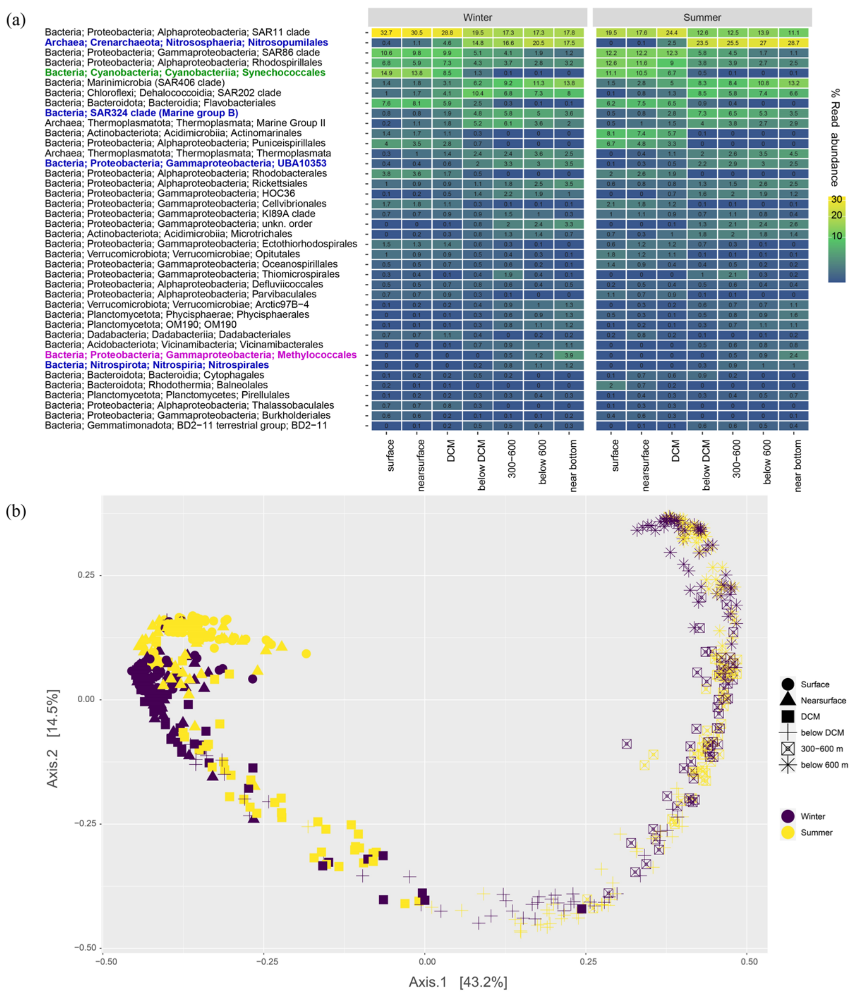

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Figure 4: (a) Read abundance of the 40 most abundant taxa (order level) from different depths offshore the Southeastern Mediterranean Sea. Surface samples are within the range of 1-5 m depth; near surface are between 20 and deep chlorophyll maximum (DCM); below DCM corresponds to 180-240 m depths; near bottom samples were taken circa 5 meters above the seafloor. Potential DCF microbes are shown in blue, Methylococcales methanotrophs are marked in magenta, and photosynthetic Synechoccocales are shown in green for reference. (b) A principal component analysis showing the differences in the structure of microbial populations (40 most abundant taxa) based on 16S rRNA gene read mapping.

4 Conclusions

Based on the conceptual model in Figure 5 that summarizes the annual microbial carbon exchanges in the southeast Mediterranean sea's offshore area, it is clear that DOC supplied by LPP is negligible and cannot explain the 'high' BCD in the area, especially in the aphotic zone that is considered a 'microbial hotspot' with high bacterial activity per cell (Hazan et al., 2018; Rahav et al., 2019). Therefore, our back of the envelope calculations highlights that DCF may provide a substantial amount of the missing carbon, at least in the southeast Mediterranean Sea, while source/s for the remaining missing carbon are currently unknown and warrant more research.

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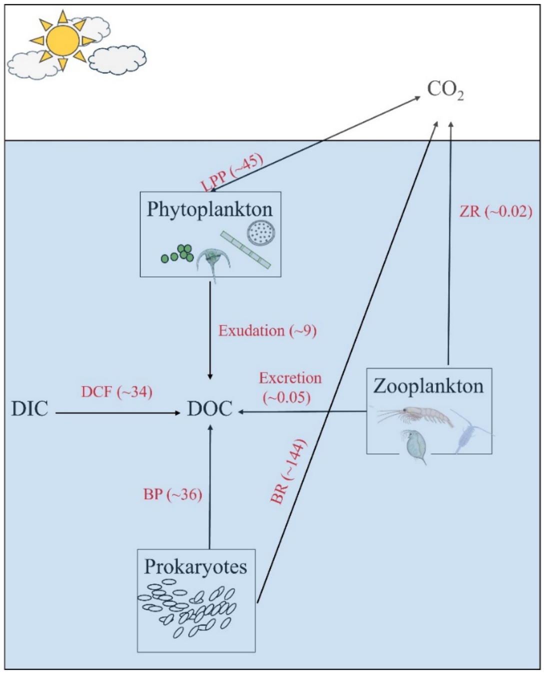

Figure 5: A schematic illustration showing microbial carbon exchange in the southeast Mediterranean Sea. Values shown are the annual averages of LPP, DCF and BP. DOC exudation from LPP was 20% of the rates. BR was calculated from BP and assuming BGE=0.20, Zooplankton's respiration (ZR) and excretion were compiled from Belkin et al. (2022). The numbers in brackets show the integrated values over 1750 m and expressed as g C m^{-2} y^{-1}.

Regardless of the yet missing DOC sources, our results demonstrate the pivotal role DCF play in compensating metabolic imbalances in carbon sources at the aphotic southeast Mediterranean Sea. Similarly, DCF was shown to be a significant process supporting microbial respiration and/or activity in aphotic layers (Baltar et al., 2009; Herndl et al., 2005; Yakimov et al., 2011), as well as in hydrothermal vents (Mattes et al., 2013) and cold seeps (Nakagawa et al., 2007).

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We note that despite the contribution of DCF to the DOC pool, as well as the other sources, very little of fixed carbon as particulate organic matter (POC) ends up in sediment traps located above the seabed (2-6%). This suggests that most of the fixed carbon arriving from DCF (as well as LPP other potential sources) is recycled in the water column and does not reach the seabed. The rapid microbial recycling of nutrients was mostly investigated in the photic layer of the southeast Mediterranean Sea (e.g., PO_4, Thingstad et al. (2005) and little is known about the processes, which are often cryptic (e.g., NO_2^- oxygenation), occurring in the aphotic layers. Our study highlights the need for adding DCF measurements to global carbon budgets. Moreover, understanding the factors affecting DCF in the photic and aphotic layers may provide science-based operational opportunities to increase atmospheric inorganic carbon sequestration.

Data availability statement

The raw physiological data used to generate figures 1-3 can be downloaded from the PANGEA repository: Reich, Tom (2025): Primal, chemo and bacterial productivity coupled with inorganic nutrient concentrations from an off shore, outgoing transect cruises. PANGAEA, https://doi.org/10.1594/PANGAEA.975231. Genomic data can be downloaded from the NCBI SRA archive project number PRJNA1215023.

Author contribution

TR: Formal experimental work and data analyses; Investigation; Data Curation; Visualization; Writing – Original Draft Preparation. NB: Resources; Writing – Review & Editing. GSV: Investigation; Writing – Review & Editing. HH:

Investigation. MRB: Investigation; Writing – Review. IBF: Conceptualization; Funding Acquisition; Supervision; data interpretation, Writing – Review & Editing. ER: Conceptualization; Formal Analysis; Funding Acquisition; Supervision; Visualization; Writing – Review & Editing.

Competing interests

The authors declare that they have no conflict of interest.

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Statements and Declarations

The authors declare that they have no conflict of interest.

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