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Inorganic carbon fixation by deep prokaryotes as an unaccounted-for CO₂ sink in Antarctic waters



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The Ross Sea is one of the most productive regions of the Ocean and is a major site of deep-water formation, ultimately affecting the global thermohaline circulation. As such, it plays a pivotal role in regulating atmospheric CO₂ through the biological and the solubility carbon pumps. Despite the recognised importance of this area, the knowledge of the carbon fluxes mediated by microbes in its interior remains limited. Here we describe the outcomes of 62 incubation experiments aimed at understanding the processing of inorganic and organic carbon pools between 200 and 2000 m. Results showed that in these waters DIC fixation occurs at rates between 0.03 and 3.12 nmol C L⁻¹ d⁻¹ and represents a relevant CO₂ sink, especially under high particulate organic carbon conditions. Both Archaea (Nitrosopumilaceae) and Bacteria (SUP05 and SAR324) were identified as putative major DIC fixers in High Salinity Shelf Water, Circumpolar Deep Water, and Antarctic Bottom Water.

A sound knowledge of the functioning of the oceanic C cycle is essential for predicting the consequences of CO₂ increase in the atmosphere. Classically, the dark ocean, which accounts for 95% of the Earth's biosphere¹, has been considered a purely heterotrophic environment dominated by the mineralization of organic matter exported from the surface layers². However, recent data suggest that the amount of CO₂ (*sensu* DIC = Dissolved Inorganic Carbon) fixed in deep ocean systems is comparable to the amount taken up by photosynthetic organisms in the illuminated part of the water column^{3–5}. Dark DIC fixation is carried out by both chemoautotrophs and heterotrophs. Chemoautotrophs in the Ocean obtain energy from the oxidation of reduced compounds such as ammonia⁶, nitrite⁷, sulphur species⁸, etc. and incorporate DIC for biomass build-up. Heterotrophs incorporate inorganic carbon through several carboxylation reactions, that are integral to their central and peripheral metabolic pathways⁹, addressed at the synthesis of fatty acids, amino acids and nucleotides as an extra source of C in oligotrophic conditions¹⁰ or to cope with pulsed inputs of organic material¹¹.

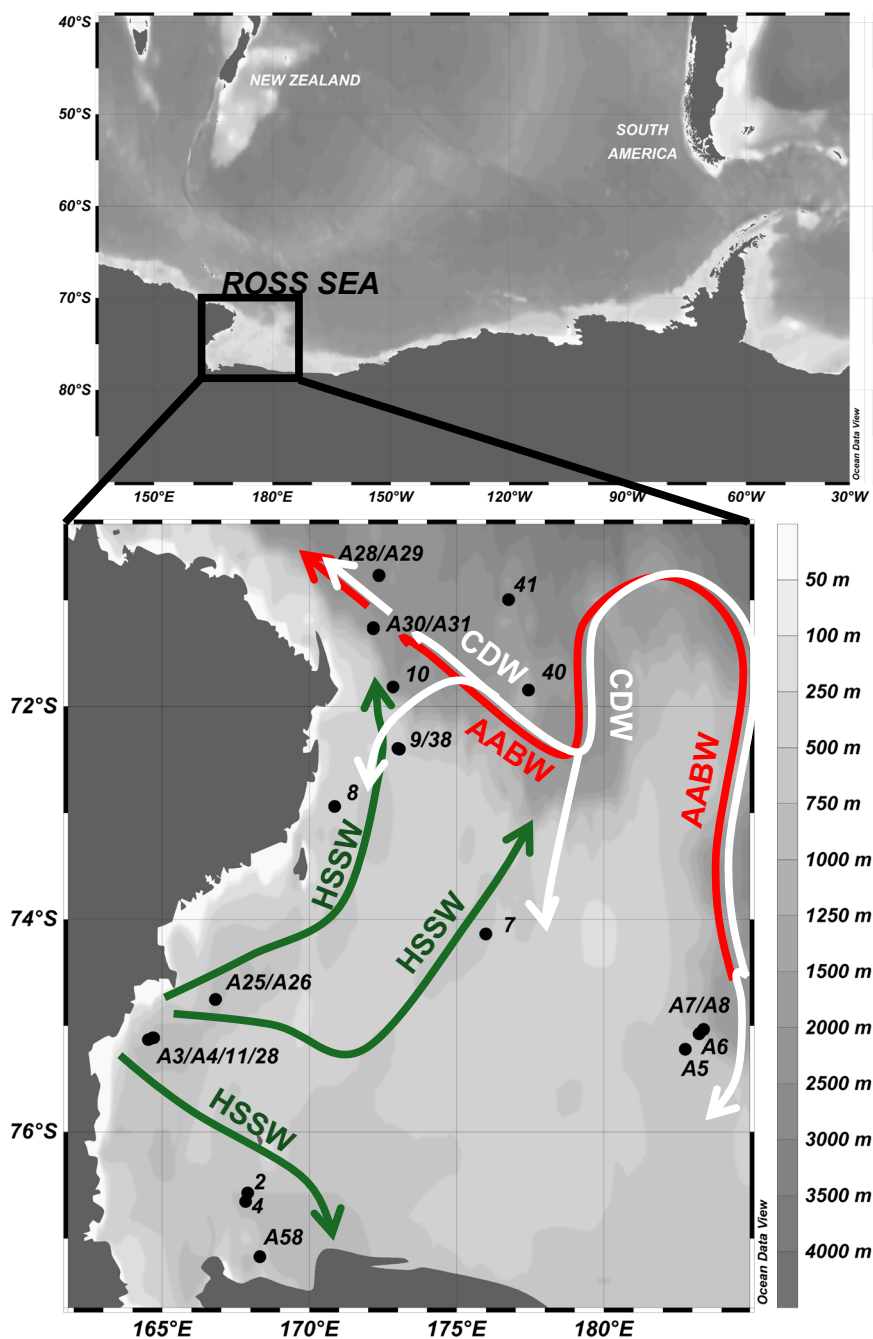
The Southern Ocean represents about 10% of the world's oceans¹², yet it plays a crucial role in ventilating the global ocean and absorbing approximately 10% of anthropogenic CO₂ emissions^{13,14}. Despite being a high nutrient-low chlorophyll system due to the limiting concentrations of critical micronutrients, and in particular iron^{15,16}, its marginal seas are

primary production hotspots¹⁷. Notably, the Ross Sea is one of the most productive regions in the Southern Ocean, contributing to one-third of its annual productivity and over 25% of its total CO₂ uptake^{18,19}. About half of the carbon fixed by surface-dwelling primary producers is exported as particulate organic matter (POM) to the mesopelagic zone, potentially accounting for up to 40% of global POM export^{2,20}. During winter, the lack of light severely limits photosynthesis and subsequent carbon export²¹; therefore, planktonic microbial communities are sustained by the Dissolved Organic Carbon (DOC) accumulated in summer and by dark DIC fixation, which corresponds to ca. 10% of total prokaryotic production²².

As a critical site of deep-water formation²³, the Ross Sea plays a pivotal role in the sequestration of CO₂ to the deep ocean. Dense shelf waters, originated mainly during winter, sink and contribute to the formation of Antarctic Bottom Water (AABW), the most voluminous water mass in the deep ocean (30–40%)²⁴, which supports the lower cell (i.e. the deep thermohaline-driven limb of the ocean conveyor belt) of the global overturning circulation²⁵. In detail, in the western sector of the Ross Sea (Fig. 1), the most important dense shelf water is High Salinity Shelf Water (HSSW), formed mainly in Terra Nova Bay, characterised by salinities >34.75, potential temperature (Θ) below –1.58 °C and rich in oxygen (ca. 280–295 μmol L⁻¹). HSSW spreads both southward toward the Ross Ice Shelf cavity and northward along the Drygalski basin toward the shelf break off Cape

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Fig. 1 | Map of the sampling stations in the Ross Sea (Southern Ocean). Station names beginning with ‘A’ were sampled in 2014; those identified by numbers only were sampled in 2016. In the lower panel the main geographical dynamics of the three water masses are reported: High Salinity Shelf Water – HSSW in green; Circumpolar Deep Water – CDW in white; Antarctic Bottom Water – AABW in red. Outside of the shelf break AABW spreads underneath CDW. The water mass circulation diagram is a simplification of the original version by Budillon et al. (2003)²³. The maps were created with the ODV software⁸⁷.



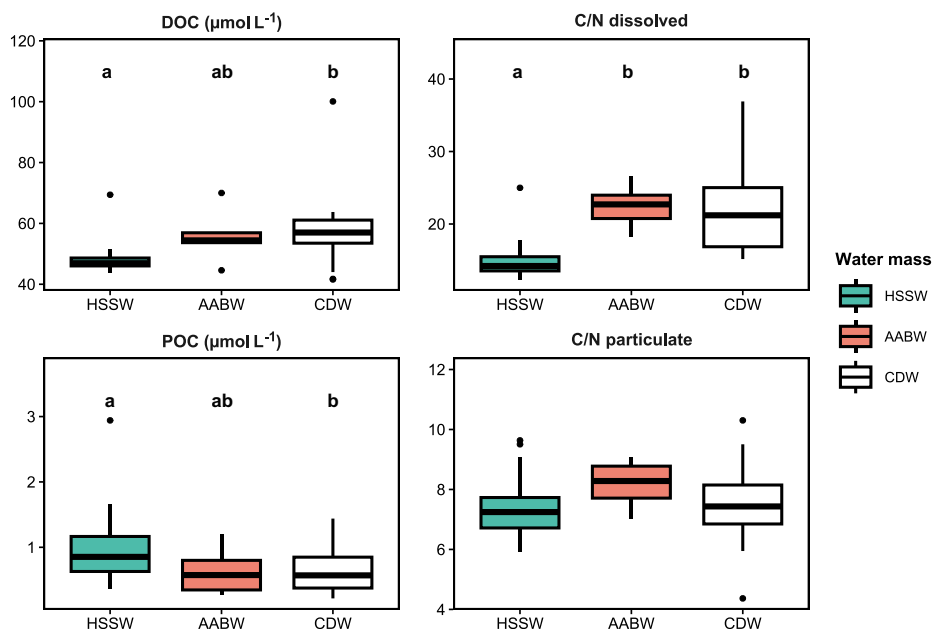
Adare where it mixes with Circumpolar Deep Water (CDW). CDW is the most voluminous water mass of the Southern Ocean; it flows westward around the continent and, in the Ross Sea area, it forms a thick layer (> 1013 m) everywhere along the slope. It is an old, warm ($\Theta > 0.6$ °C), oxygen-poor (ca. $190\text{--}200$ $\mu\text{mol L}^{-1}$) and nutrient-rich water mass (NO_3^- : $\sim 30\text{--}33$ $\mu\text{mol L}^{-1}$; PO_4^{3-} : $\sim 2\text{--}2.5$ $\mu\text{mol L}^{-1}$). The mixing of CDW and HSSW generates AABW, a deep water mass with a neutral density > 28.27 kg m^{-3} , which holds intermediate biogeochemical features^{26–28}.

Despite the cornerstone role that the Southern Ocean plays in the global carbon budget, very little information is available on the biodiversity and metabolism of the deep microbiome in its marginal seas and in the Ross Sea in particular.

In this work, we discuss 62 incubation experiments performed during two summertime cruises in the western Ross Sea which aimed at understanding the processing of inorganic and organic C pools in HSSW, CDW

and AABW between ca. 200 and 2000 m depths (Fig. 1; Supplementary Table 1). We investigated the DIC uptake (by chemosynthesis or anaplerosis) and production (by respiration), as well as the utilization of dissolved organic carbon (by heterotrophic production) and its release (by excretion or viral lysis). Our overarching hypothesis was that in CDW, the oldest, oxygen-poor water mass, chemoautotrophy could help supply microbial communities with freshly-produced organic carbon, whereas in the youngest, POM-rich HSSW heterotrophic processes would predominate. Results showed that in these waters DIC fixation occurs at rates comparable to those measured in most oceanic regions and could account for 5% of the currently estimated yearly CO_2 sink in the Ross Sea. Higher DIC fixation occurred in high POM conditions, thus suggesting a priming effect of organic particles. Autotrophic/mixotrophic prokaryotes were abundant and evenly distributed in the three water masses, suggesting that chemolithotrophic pathways do not prevail in one specific water mass, but are ubiquitous to all three.

Fig. 2 | Organic matter concentration and features of the three water masses. Box-whiskers plots [box = interquartile range with median; whiskers = range without outliers] of Dissolved Organic Carbon (DOC) concentration, of the ratio between organic C and N in the dissolved phase, of the Particulate Organic Carbon (POC) concentration and of the ratio between organic C and N in the particulate phase in High Salinity Shelf Water (HSSW, left boxes), Antarctic Bottom Water (AABW, central boxes) and Circumpolar Deep Water (CDW, right boxes). Letters above boxes refer to results of the Kruskal-Wallis ANOVA, followed by the post hoc Dunn's test ($p < 0.05$). Different letters indicate significant differences among water masses.



Results and discussion

Environmentally driven metabolism of deep-sea microbes in the Ross Sea

Our survey was conducted during two summer cruises and, in both expeditions, samples were collected in High Salinity Shelf Water, Circumpolar Deep Water and Antarctic Bottom Water. The biogeochemical setting did not display substantial interannual variability, with few exceptions regarding POC, PN, NO_3^- and NH_4^+ whose concentrations were slightly lower during the first cruise in CDW and AABW (Supplementary Fig. 1). Inter-annual variations in the water masses' chemistry have already been described and attributed mainly to differences in yearly HSSW formation, and consequent differential mixing with CDW at the shelf break²⁷. Overall, CDW was enriched in NO_3^- ($33.43 \pm 0.76 \mu\text{mol L}^{-1}$) and PO_4^{3-} ($2.02 \pm 0.12 \mu\text{mol L}^{-1}$) if compared to HSSW (31.35 ± 0.56 and $1.97 \pm 0.07 \mu\text{mol L}^{-1}$, respectively) (Supplementary Fig. 2). NH_4^+ concentration did not vary among water masses (overall mean $\pm$ SD = $0.17 \pm 0.08 \mu\text{mol L}^{-1}$), whereas the highest NO_2^- load was observed in AABW ($0.04 \pm 0.01 \mu\text{mol L}^{-1}$). Important differences among water masses were observed in the organic matter pool. In fact, samples collected in HSSW were richer in POC ($0.97 \pm 0.50 \mu\text{mol L}^{-1}$ in HSSW vs. $0.64 \pm 0.34 \mu\text{mol L}^{-1}$ in CDW), poorer in DOC ($48.37 \pm 6.04 \mu\text{mol L}^{-1}$ in HSSW vs. $58.56 \pm 12.62 \mu\text{mol L}^{-1}$ in CDW) and their C/N ratio indicated a more bioavailable pool of dissolved organic matter²⁹ (15.16 ± 3.12 in HSSW vs. 24.73 ± 11.24 in CDW (Fig. 2). In fact, the stoichiometry of the organic matter pool, and in particular the ratio between C and N is indicative of the bioavailability or the age of substrates, since proteinaceous, N-rich molecules are utilised more quickly by degrading microbes, resulting in an enrichment of C-rich compounds, both in the particulate³⁰ and the dissolved²⁹ pools. Altogether, these results reflect the high biological productivity of HSSW formation areas¹⁹, where pronounced organic matter synthesis by phytoplankton reflects in high export of particulate organic matter²⁰, and sinking particles are subjected to a continuous release of dissolved compounds by the hydrolytic activity of particle-attached microbes³⁰.

In such an environmental scenario, microbial activities were generally enhanced in HSSW if compared to CDW, being influenced by the organic matter pool and features (Fig. 3 and Supplementary Fig. 3). In particular, both Heterotrophic C Production (HCP) and respiration rates were highest in HSSW (HCP: $33.25 \pm 16.29 \text{ nmol C L}^{-1} \text{ d}^{-1}$ in HSSW vs. $1.86 \pm 1.34 \text{ nmol C L}^{-1} \text{ d}^{-1}$ in CDW; Respiration with 0.68 conversion factor: $3.70 \pm 1.88 \text{ nmol C L}^{-1} \text{ d}^{-1}$ in HSSW vs. $16.71 \pm 9.33 \text{ nmol C L}^{-1} \text{ d}^{-1}$ in CDW) and correlated positively with POC and negatively with dissolved C/N

(Spearman's ρ : POC vs HCP = 0.54, $N = 62$, $p < 0.001$; POC vs Respiration = 0.53, $N = 61$, $p < 0.001$; C/N vs HCP = -0.40 , $N = 36$, $p = 0.017$; C/N vs Respiration = -0.37 , $N = 36$, $p = 0.027$). Results about these metabolic features are subjected to the choice of conversion factors (CF). While the Leucine-to-Carbon CF in the deep-sea literature can vary by approximately two-fold³¹, CFs for computing respiration rates with the ETS method can span two orders of magnitude³². In this study for HCP we adopted the commonly used $1.55 \text{ kg C mol}^{-1}$ leucine in order to be able to compare our results with most literature reports. For respiration, two different CFs were used (see 'Methods'), being the ones that resulted in the most likely prokaryotic growth efficiency according to literature findings³³ (Supplementary Fig. 4). Bearing these caveats in mind, in this study our purpose was to establish a comparison among the water masses, therefore we show both alternatives for rates calculations (Fig. 3, Supplementary Fig. 4).

DIC uptake rates ranged between 0.03 ± 0.00 and $3.12 \pm 0.49 \text{ nmol C L}^{-1} \text{ d}^{-1}$ (Fig. 3) and highlighted values comparable to those assessed elsewhere in the world's oceans (Table 1) despite the lower temperatures. Notably, we were not able to detect a measurable release of newly produced organic matter (excreted radiolabelled DOC), differently from what has been observed in culture³⁴ and in the deep Mediterranean Sea^{3,35}. The very few reported values of dark DIC fixation for Antarctic waters^{22,36,37} are consistent with the data presented here (Table 1). Higher rates have been observed in association with the McMurdo Ice Shelf cavity ($6.0 \pm 1.0 \text{ nmol C L}^{-1} \text{ d}^{-1}$)³⁶, which is inhabited by abundant and active chemoautotrophic organisms³⁸. On average ($\pm$ SD), the ratio between DIC uptake and HCP was 0.21 ± 0.16 in AABW, 0.28 ± 0.22 in CDW, and 0.38 ± 0.20 in HSSW, in line with values computed in several water masses of the western basin of the North Atlantic³⁹.

Interestingly, as observed for HCP and respiration, DIC uptake in HSSW was higher (three-fold on a median basis) than rates measured in the other water masses (Fig. 3).

Particles in the ocean are hotspots of microbial activities due to their high content of inorganic nutrients and organic substrates (e.g. Azam and Long, 2001³⁰); therefore, it is not surprising that higher heterotrophic activities are measured in systems with high POC content³⁷. However, high rates of DIC fixation in the deep ocean have been attributed to two contrasting mechanisms. In the first, the highest inorganic carbon fixation rates are observed as a direct upregulation in response to the scarcity of the DOM pool^{3,40}. The second mechanism sees a priming effect by specific organic compounds which trigger this metabolic trait and the upregulation of assimilatory carboxylases involved in amino acids, nucleic acids precursors,

Fig. 3 | Microbial activities in the three water masses. Box-whiskers plots [box = interquartile range with median; whiskers = range without outliers] of Dissolved Inorganic Carbon (DIC) fixation rates, Respiration rates measured by the ETS method using the 0.68 conversion factor (CF), Heterotrophic Carbon Production rates and Prokaryotic Growth Efficiency, in High Salinity Shelf Water (HSSW, left boxes), Antarctic Bottom Water (AABW, central boxes) and Circumpolar Deep Water (CDW, right boxes). Letters above boxes refer to results of the Kruskal-Wallis ANOVA followed by the post hoc Dunn's test ($p < 0.05$). Different letters indicate significant differences among water masses.

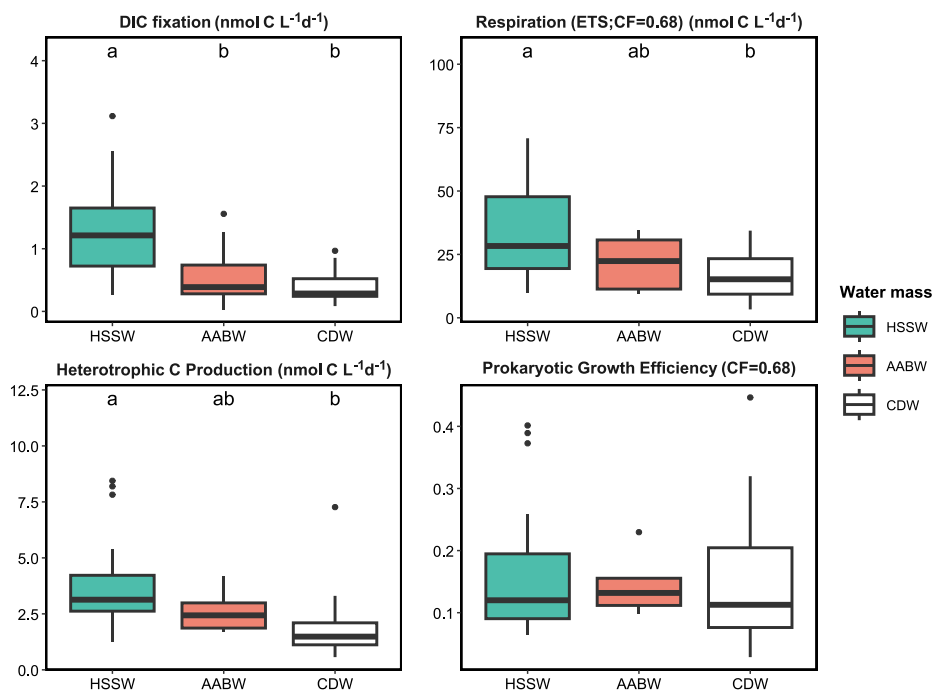


Table 1 | DIC fixation rates measured in the major ocean basins at depths greater than 200 m

Ocean	Region	DIC fixation rates	Reference
		nmol C L ⁻¹ d ⁻¹	
Arctic	Eastern Fram Strait	0.08–24.04	52
Atlantic	Central-North Atlantic	0.19–5.36	39
	Santos Basin	4–2200	82
	Subtropical NE Atlantic	12 ± 5	83
Indian	Arabian Sea	0.5–6	84
Pacific	Eastern tropical/subtropical Pacific	0.3–1.9	85
Mediterranean Sea	Tyrrhenian Sea	4.16–19.16	86
	whole basin	0.57–240.42	3
Southern	Southern Drake Passage	0.01–3.33	22
	Ross Sea	0.20–0.71 ^a	37
	Ross Sea	0.03–3.12	This study
	Ross Sea, McMurdo Ice Shelf cavity	6.0 ± 1.0	36

^aMeasured on the 0.2–1 μm fraction.

and fatty acids biosynthetic pathways^{11,37,41}. Our data suggest a third mechanism, where DIC fixation is associated with the POC load or export (Spearman's $\rho = 0.51$, $N = 61$, $p < 0.001$). So far, pronounced anaplerotic reactions in the deep sea have been considered less important than autotrophy due to the limited availability of organic carbon⁵. However, the exceptional particulate matter concentration resulting from the export of high surface water productivity typical of the Ross Sea¹⁹, might promote DIC uptake, especially in HSSW, possibly through heterotrophic/mixotrophic pathways⁴⁰, as observed with labile organic compounds in Atlantic mesopelagic waters¹¹. Likewise, a higher bioavailability of the dissolved organic matter pool in HSSW (as inferred by the C/N ratio) might be responsible for enhanced DIC utilization in this water mass (Spearman's $\rho = -0.52$, $N = 35$, $p = 0.001$). It was also interesting to observe that the prokaryotic growth

efficiency computed for samples collected in the different water masses did not diverge significantly (Fig. 3). In the last couple of decades emerging evidence has indicated that, notwithstanding steep environmental gradients (e.g., vertical gradients in the water column³³), metabolic rates of individual cells are likely to display only small variations. Indeed, our dataset corroborates these findings (Supplementary Fig. 5), showing similar per cell activities in the three water masses despite significant differences in prokaryotic abundance (Fig. 3, Supplementary Figs. 2 and 3). Similar to our finding, in the North Atlantic Ocean it has been reported that higher prokaryotic abundances were characteristic of newly formed, particle-rich water masses³⁹; therefore, we speculate that in the Ross Sea high-quality organic matter sustains a more abundant prokaryotic population in HSSW, whereas recalcitrant resources, coupled with higher relative abundances of predators⁴², would be responsible for lower cell numbers in CDW. This picture would, ultimately, be responsible for the differences observed in metabolic rates between HSSW and CDW. Antarctic Bottom Water, being generated by the mixing of the aforementioned water masses, displayed intermediate biogeochemical and microbiological features (Figs. 2 and 3; Supplementary Figs. 2 and 3).

Taken together, our dark DIC fixation data account for 1% of surface photosynthetic primary production data during summer (e.g. 4–8 μmol C L⁻¹ d⁻¹)⁴³. However, not being limited by light availability, DIC fixation occurs below 200 m all year round. Therefore, when considering the volume of AABW (2470 km³), HSSW (71,580 km³) and CDW (173,140 km³)²⁶ and the mean DIC fixation rates per water mass (0.58, 1.30 and 0.41 nmol C L⁻¹ d⁻¹, respectively), we could assess that the dark portion of the Ross Sea is responsible for a DIC sequestration rate of ca. 2 Gg C d⁻¹ (0.7 Tg C yr⁻¹) (Supplementary Table 2). This rate is equivalent to ca. 5% of the estimated CO₂ sink in the Ross Sea (13 Tg C yr⁻¹)¹⁸, thus representing a non-negligible, previously unaccounted carbon sink.

Putatively autotrophic microbes in the Ross Sea deep water masses

Given the high contribution of DIC fixation to total carbon utilization by prokaryotes in the deep Ross Sea, and aiming to identify the major players of such processes, we investigated the biodiversity of deep-sea microbes using a metabarcoding approach targeting the generic prokaryotic barcode V4-V5⁴⁴, and specific regions for Bacteria⁴⁵ and Archaea⁴⁶. Although the

knowledge of the deep-sea microbiome is far from being fully dissected, there is some information on the most abundant microorganisms living in it and, to a minor extent, on their biogeochemical relevance. For this reason, we have specifically searched for Archaea and Bacteria known for their chemoautotrophic potential. Archaea of the Nitrosopumilaceae family, in particular, are thought to contribute to a large fraction of DIC fixation in the deep ocean^{5,47}. Likewise, Bacteria belonging to the phylum Nitrospinae, the SAR324, SUP05 and Arctic97B-4 groups, among others, have been regarded as important chemoautotrophs in oceanic depths^{7,48,49}.

An initial β -diversity analysis of the bacterial assemblages showed that the different water masses harboured specific communities and that the

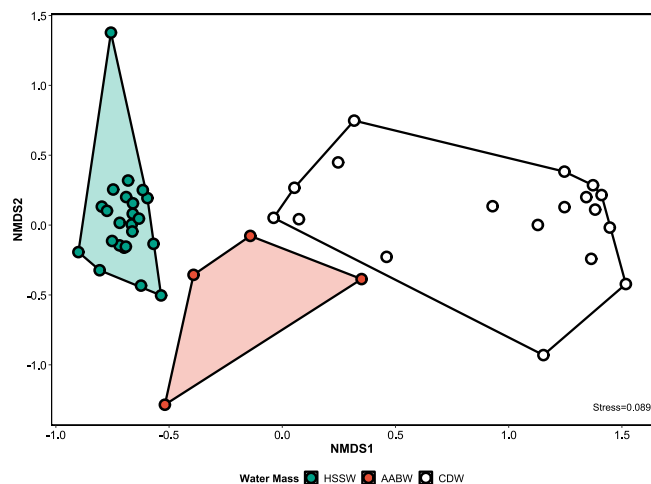


Fig. 4 | Non-metric multidimensional scaling (nMDS) performed on the 16S rRNA bacterial gene dataset using a Jaccard dissimilarity matrix and average linkage approach. Samples collected in High Salinity Shelf Water (HSSW) are reported in green, those collected in Antarctic Bottom Waters (AABW) are reported in red and those collected in Circumpolar Deep Water (CDW) are reported in white. Convex hulls group together samples from specific water mass and are colour-coded accordingly.

AABW, formed by the mixing of CDW and HSSW, retained signatures of both parental water masses (Fig. 4). This phenomenon is well documented both in polar seas^{50–52} and at lower latitudes^{35,53}. As water masses are characterized by specific physical and biogeochemical features, they exert bottom-up pressures on the selection of certain taxa⁵⁴; in addition, since the peculiar biogeochemical fingerprint of a water mass is likely to be preserved during advection and convection processes, microbial communities are transported over long distances with minor perturbations^{51,55,56}. In our dataset, Archaea represented a strikingly constant presence within the communities of all water masses at the domain level ($25.9 \pm 7.0\%$ relative abundance, Supplementary Fig. 6).

Nevertheless, the different water masses harboured distinct relative abundance of specific ASVs belonging to the Nitrosopumilaceae family that collectively comprised roughly half of the whole archaeal community (on average 53.9 ± 6.4 , Supplementary Fig. 7). Such a consistent representation of archaeal nitrifiers in our samples suggests that different water masses have a similar potential for DIC fixation associated to ammonia oxidation, although we cannot exclude that some taxa (ASVs) might be more efficient than others in autotrophic processes³⁴. Moreover, several studies have pointed out that ammonia oxidation by Archaea cannot explain the high DIC fixation rates in the ocean's interior alone^{39,57}. However, it has been reported that underneath the Ross Ice Shelf, archaeal ammonia oxidisers are among the most active DIC fixers, as revealed by metatranscriptomic analyses³⁸. We further focused on the composition of chemoautotrophic bacteria involved in the oxidation of nitrite and reduced sulphur species (Fig. 5). Among these, the most abundant taxa were the SUP05 and the SAR324 clusters. Members of the clade SUP05 can live autotrophically by oxidising hydrogen and reduced sulphur species (elemental sulphur, sulphide, thiosulfate) and fixing inorganic carbon⁸. This cluster (Thioglobaceae family) is widely distributed in deep water masses^{48,56,58}, and is often found in oxygen-depleted zones, in hydrothermal vent-influenced areas⁸ and in low-light, organic carbon-poor conditions⁵⁹. In our study, this cluster represented a relevant proportion of the bacterial communities, showing higher relative abundance (Fig. 5; Supplementary Table 3) in CDW ($11.38 \pm 2.92\%$) than in HSSW ($6.24 \pm 2.85\%$). Bacteria closely related to the SUP05 cluster (UBA868) have recently been reported as the most active microbes transcribing RuBisCO and dominating the transcription of genes

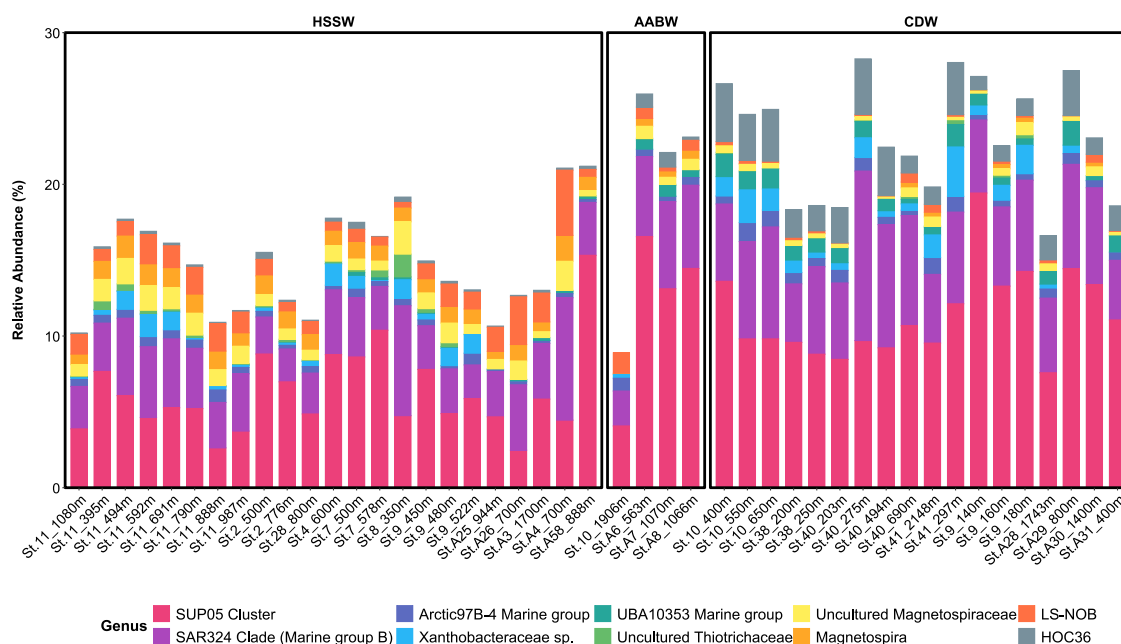


Fig. 5 | Bar plot of the relative abundance of putative chemoautotrophic bacterial taxa. Relative abundances are reported at the highest possible taxonomic resolution assigned with the Silva database. Labels indicate station_depth.

Significant differences between the relative abundance of taxa in High Salinity Shelf Water (HSSW) and Circumpolar Deep Water (CDW) are highlighted in Supplementary Table 3.

indicative of sulphur oxidation⁶⁰. Oxygen concentration in the water column throughout the Ross Sea is unlikely to promote the formation of H₂S; therefore, the SUP05 bacteria must obtain the energy for autotrophic processes from thiosulfate, S₀ or hydrogen. Likewise, SAR324 is a key taxon in the dark ocean microbiome worldwide, showing high abundance, a notable metabolic versatility, and the gene repertoire for inorganic carbon fixation⁶¹. Notwithstanding the lack of cultured representatives, DNA-based studies indicate that this cluster can obtain energy from the oxidation of thiosulfate^{48,61} or from the oxidation of sulphite generated by taurine degradation in oxygenated waters⁶², being one of the most transcriptionally active taxa in the deep-sea microbiomes⁶³. In our dataset, the relative abundance of this taxon was higher in CDW than in HSSW, being on average ($\pm$ SD) 6.05 $\pm$ 1.75% and 3.79 $\pm$ 1.46%, respectively (Fig. 5; Supplementary Table 3).

Even when considering all the putative chemolithotrophic taxa, we observed that the newly-formed HSSW harboured a slightly minor (relative) abundance of bacteria able to fix DIC autotrophically (14.97 $\pm$ 3.23% in HSSW vs 22.95 $\pm$ 3.84% in CDW).

Altogether, our data indicate pronounced DIC fixation rates in deep water masses of the Ross Sea and highlight several microbial taxa as potentially responsible for such processes. Whether this fixation is imputable primarily to autotrophic or to anaplerotic processes is still to be determined, even though under organic matter-limited conditions chemosynthetic microbes were shown to play a pivotal role⁵. Further studies involving the genetic repertoire of deep microbiomes are needed to shed more light on such processes.

Methods

Sampling strategy and environmental setting

The sampling stations (Fig. 1) were selected based on previous investigations where the target water masses had been identified^{23,50,64}. The sampling was performed during the XXIX and the XXXI Italian expeditions in Antarctica onboard the R/V *Italia* in early 2014 and 2016. Full-depth hydrographic casts and water sampling were carried out using a SBE 9/11 PLUS CTD, with dual temperature and salinity sensors mounted on a SBE 32 carousel sampler equipped with twenty-four 12-L Niskin bottles. Sampling depths were chosen according to the physical structure of the water column (salinity and potential temperature data). Details on sampling stations are reported in Supplementary Table 1; water masses were identified according to the physical features described in Budillon et al. (2003)²³. At each selected depth, seawater was collected for the following analytical determinations: inorganic macronutrients, dissolved and particulate organic carbon and nitrogen, prokaryotic abundance, heterotrophic carbon production (HCP), planktonic respiration (ETS), dissolved inorganic C (DIC) uptake and prokaryotic diversity.

Dissolved inorganic nutrients (PO₄³⁻, NH₄⁺, NO₂⁻ and NO₃⁻), Dissolved Organic C (DOC) and particulate organic matter (C = POC; N = PN) concentrations were determined by standard procedures as described in Quero et al. (2020)⁵². Total dissolved nitrogen was estimated as NO₃⁻ after wet persulfate oxidation, according to Hansen and Koroleff (1999)⁶⁵ and the organic fraction was calculated as the difference between the total and the inorganic dissolved N concentration.

Microbial abundance, activity and diversity

Total prokaryotic abundance was measured by epifluorescence microscopy in 2014 and by flow cytometry in 2016, as described by Ingrosso et al. (2022)²⁸ and Manna et al. (2020)³⁷, respectively. In the first case, samples were preserved using a 0.2- μ m filtered formaldehyde solution (2% final concentration, f.c.; Fluka), then stained with a 4',6-diamidino-2-phenylindole (DAPI) solution at 1 μ g mL⁻¹ f.c., in the dark for 15 min. Cells were subsequently collected on 0.22- μ m black polycarbonate filters (Nucleopore) and enumerated with an Olympus BX 60 F5 microscope using a UV filter set (BP 330–385 nm, BA 420 nm) at a final magnification of 1000 $\times$. Three analytical replicates (3–8 mL each) were processed for each sample; for every replicate, a minimum of 200 cells were counted in at least 20 randomly

selected fields. For flow cytometry analyses, 1.7 mL seawater aliquots were fixed with glutaraldehyde (0.5% f.c., Grade I for EM analyses, Sigma-Aldrich). Samples were kept at 4 °C for approximately 15 min and then stored at –80 °C until analysis. Before enumeration, samples were thawed at room temperature and diluted 1:10 with 0.2- μ m filtered 1 $\times$ Tris-EDTA buffer (Sigma-Aldrich). They were then stained with SYBR Green I nucleic acid dye (1 $\times$ f.c., Life Technologies) for 10 min in the dark at room temperature. Analyses were performed using a FACSCanto II flow cytometer (Becton Dickinson) equipped with an air-cooled 488 nm laser and standard filter configuration. Samples were run for 2 min at a flow rate of approximately 60 μ L min⁻¹, with green fluorescence used as a trigger. Data acquisition and processing were carried out with the FACSDiva software (Becton Dickinson). The flow rate was calibrated daily by running distilled water and determining its mass before and after analysis (at least 5 replicates). Abundances were calculated based on the recorded counts and the corresponding flow rates. Heterotrophic carbon production (HCP) was determined from the incorporation of ³H-leucine⁶⁶. Triplicate 1.7 mL aliquots and one killed control (amended with 90 μ L of 100% trichloroacetic acid) were spiked with 20 nM radiotracer^{67,68} (50.2 Ci mmol⁻¹; Perkin Elmer) and incubated in the dark for 6 h at in situ temperature. The extraction of ³H-labelled proteins was performed using the microcentrifugation protocol⁶⁹. Radioactivity was measured with a TRI-CARB 2900 TR Liquid Scintillation Analyzer following the addition of 1 mL of scintillation cocktail (Ultima Gold™ MV; Packard). Carbon production was calculated using a conversion factor of 1.55 kgC mol⁻¹ Leu incorporated, assuming no internal isotope dilution⁷⁰.

Planktonic respiration was estimated by the Electron Transport System activity method, following Packard and Williams (1981)⁷¹ as detailed by Reinthaler et al. (2006)⁷² with slight modifications. Briefly, 16 L of seawater, prefiltered through 200 μ m mesh were further filtered onto precombusted GF/F filters, which were then stored at –80 °C until analysis. In the laboratory, filters were transferred to polypropylene tubes containing 4 mL of phosphate buffer. Samples were kept on ice and subjected to ultrasonication (Ultrasonic Processor XL, Misonix) for 15 s, followed by manual homogenization using a glass Potter for 2 min. The homogenate was centrifuged at 1810 $\times$ g for 10 min at 4 °C. Subsequently 1 mL of the supernatant was incubated at 15 °C with 3 mL of substrate solution (NADH, NADPH and sodium succinate) and 1 mL of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT). Two aliquots (1 mL each) of supernatant combined with substrate solution were used as analytical replicates. After 20 min, the reaction was terminated by adding 1 mL of a formalin-sodium formate mixture. All solutions were freshly prepared. The absorbance was measured at 490 nm using a Varian Cary 100 SCAN spectrophotometer. Duplicate GF/F filters rinsed with sterile MilliQ water were used as controls. ETS activity was measured as follows:

$$\text{ETS } (\mu\text{L O}_2 \text{ L}^{-1} \text{ h}^{-1}) = (60 \times S \times H \times OD_{\text{corr}}) / (1.42 \times f \times V \times t)$$

where 60 converts minutes to hours, *S* is the volume of the quenched reaction mixture (in mL), *H* is the volume of the homogenate (in mL), *OD_{corr}* is the absorbance of the sample corrected with the absorbance of blanks, 1.42 converts INT-formazan formed to oxygen units, *f* is the volume of the homogenate used in the reaction, *V* is the volume of the seawater sample, *t* is the reaction time. ETS activity was corrected to in situ temperature using the equation:

$$\text{ETS}_{\text{INSITU}} (\mu\text{L O}_2 \text{ L}^{-1} \text{ h}^{-1}) = \text{ETS} \times e^{[-K1/R \times (1/T1 - 1/T2)]}$$

where *K1* is the activation energy (15.8 Kcal mol⁻¹), *R* is the gas constant, *T1* and *T2* are the temperatures (in °K) in situ and in the assay, respectively. Two Respiration/ETS conversion factors were compared to compute respiration rates, calculated from ETS_{INSITU} assuming a respiratory quotient = 1. The conversion factors were 0.68⁷³ and 5.3⁷². These calculations led to prokaryotic growth efficiency [PGE = HCP/(HCP+Respiration)] values equal to 18 $\pm$ 16 and 4 $\pm$ 8 % (average $\pm$ SD), respectively.

Rates of Dissolved Inorganic Carbon (DIC) fixation were quantified by the incorporation of $\text{NaH}^{14}\text{CO}_3$ as described in Celussi et al. (2017)³. Duplicate seawater subsamples and one control (40 mL) were collected from Niskin bottles into 50 mL sterile Falcon tubes and amended with 100 μL of a $\text{NaH}^{14}\text{CO}_3$ solution (46.8 mCi mmol^{-1} ; DHI) yielding a final activity of 0.25 $\mu\text{Ci mL}^{-1}$. The control was fixed with dolomite-buffered formalin (2% f.c.). Samples were incubated in the dark for 96 h at in situ temperature and subsequently fixed with 2% dolomite-buffered formalin. The entire volume of each replicate was filtered through 0.2 μm polycarbonate membranes (Whatman), and 10 mL of the filtrate was collected and immediately frozen at -20°C . Filters were rinsed twice with 10 mL of an autoclaved NaCl solution (3.8% w/v), acidified with HCl fumes in scintillation vials for 12–16 h, and stored at -20°C . In the laboratory, scintillation vials were filled with 5 mL of scintillation cocktail (Filter-Count™, Perkin Elmer), and radioactivity was measured by a TRI-CARB 2900 TR Liquid Scintillation Analyzer. Filtrates from each replicate and control were thawed, and 5 mL aliquots were acidified with 200 μL of HCl 0.5 N for 12 to 16 h to remove inorganic carbon ($\text{pH} < 2$). Subsequently, 10 mL of scintillation cocktail (Ultima Gold™ XR, Perkin Elmer) was added prior to activity measurement. The filtration and the acidification steps were completed within 24 h of formalin fixation.

DIC fixation was determined according to the following equation⁷⁴:

$$\text{DIC fixation} = (\text{DIC} \times \text{DPM}_{\text{sample-control}} \times 1.05 \times 12) / (\text{DPM}_{\text{added}} \times T)$$

where DIC is the concentration of dissolved inorganic carbon in samples (range = 2.29–2.30 mmol L^{-1}) determined by Ingrosso et al. (2022)²⁸, 1.05 is the correction factor for slower assimilation of ^{14}C than ^{12}C , 12 is the molecular weight of C, $\text{DPM}_{\text{sample-control}}$ are DPM measured in every replicate corrected for the ones in the control, $\text{DPM}_{\text{added}}$ is the radioactivity (certified by the provider) of the $\text{NaH}^{14}\text{CO}_3$ solution added in each tube, T is incubation time. The activity in the filtrate was not measurable in any sample; therefore, we report the particulate DIC fixation rates only ($> 0.2 \mu\text{m}$).

For prokaryotic community analysis, seawater samples (5.5 L) were filtered onto 0.22 μm pore size cellulose nitrate membrane filters (Sartorius) and stored at -20°C until processing. DNA was extracted from each filter using the PowerWater™ DNA Isolation kit (QIAGEN), according to the manufacturer's instructions with a few extra steps to increase the DNA yield and quality³⁵. The DNA concentration was measured using a Qubit Fluorometer (Thermo-Fisher), and the DNA was stored at -80°C until sequencing.

For the DNA metabarcoding analysis, a combination of three barcodes was used. In all samples (45, Supplementary Table 1), the V3–V4 region of the 16S rRNA gene was amplified with the 341F and 785R universal bacterial primers⁴⁵. Then, in order to deepen the taxonomic coverage of some marine groups and to estimate the proportion of Bacteria and Archaea^{44,75}, on a selected sample subset (17, Supplementary Table 1), the V4–V5 region of 16S rRNA gene was amplified using 515-Y and 926R primers⁴⁴ and, to increase the Archaea taxonomic resolution, the same region was also amplified with 517F (five oligos in the same proportion) and 958R primers⁴⁶. Libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Illumina protocol and run on an Illumina MiSeq System for a read length of $2 \times 250 \text{ bp}$ at the COGENTECH facility (Consortium for Genomic Technologies, Milan, Italy) and BMR Genomics S.r.l., Padua, Italy (www.bmr-genomics.it).

For each barcode, primers and adapter sequences were removed from raw reads with Cutadapt v. 4.5⁷⁶. Paired-end reads were then imported and analysed in RStudio v. 4.4.0⁷⁷ using the DADA2 package v. 1.32⁷⁸. Quality check and trimming of the reads were performed following the package instructions. Paired-end reads were subsequently grouped in amplicon sequence variants (ASVs); chimeric sequences were identified and removed from the dataset. Finally, taxonomy was assigned using a native implementation of the naive Bayesian classifier method against the SILVA database v138.1⁷⁹. ASVs belonging to Eukarya, mitochondria, chloroplasts,

and with frequency < 2 (singletons) were removed. All sequences have been submitted to the SRA Sequence Read Archive at NCBI (BioProject Accession no. PRJNA1184697, PRJNA1184714, PRJNA1196803).

Estimates of DIC fixation rates in the Ross Sea

In order to calculate the regional dark DIC fixation rates, we utilised the water masses volumetric data reported by Orsi and Wiederwohl (2009)²⁶ for the Ross Sea: Antarctic Bottom Water = 2,470 km^3 ; High Salinity Shelf Water = 71,580 km^3 ; Circumpolar Deep Water (+ its modified counterpart MCDW) = 173,140 km^3 . These three volumes were multiplied by the average DIC fixation rate computed for each water mass: Antarctic Bottom Water = 0.58 $\text{nmol C L}^{-1} \text{ d}^{-1}$; High Salinity Shelf Water = 1.29 $\text{nmol C L}^{-1} \text{ d}^{-1}$; Circumpolar Deep Water = 0.41 $\text{nmol C L}^{-1} \text{ d}^{-1}$ (Supplementary Table 1).

Statistical analyses

The Shapiro-Wilk test was used to assess the normality of datasets. Significant differences in the datasets characterizing the different water masses were assessed through the Kruskal-Wallis ANOVA, followed by a post hoc 'Multiple comparisons of mean ranks for all groups' (Dunn's) test, Bonferroni corrected (Statistica v.7, StatSoft).

Non-metric multidimensional scaling (nMDS) was performed on the 16S rRNA bacterial gene dataset using a Jaccard dissimilarity matrix and average linkage approach and plotted with the ggplot2 package (v. 3.5.1)⁸⁰ in RStudio v. 4.4.0⁷⁷. Water mass-based partitioning of 16S data was tested with PERMANOVA, using the same dissimilarity distance built for the nMDS ordination and computed through the function *adonis2* of the *vegan* package (v. 2.7-1)⁸¹.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All the physical, chemical and microbiological data are available in Supplementary Table 1 and at <https://doi.org/10.13120/9vk3-0t31>. All DNA sequences have been submitted to the SRA Sequence Read Archive at NCBI (BioProject Accession nos. PRJNA1184697, PRJNA1184714, PRJNA1196803).

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Author contributions

M.C. conceived the study. M.C. and G.M.L. contributed to the sampling and experimental designs. M.C., G.M.Q., E.B. and M.B. performed laboratory analyses. G.M.Q., E.B. and M.B. conducted metabarcoding and bioinformatic analyses. M.C., V.M., G.B., P.C., A.P.R. and G.M.L. contributed to data interpretation. M.C., V.M. and M.B. data visualization. M.C. drafted the first version of the manuscript. All authors contributed to the manuscript preparation in its final version.

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Competing interests

The authors declare no competing interests.

Additional information

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