

Expanding success in the isolation of abundant marine bacteria after reduction in grazing and viral pressure and increase in nutrient availability

Xavier Rey-Velasco,¹ Ona Deulofeu-Capo,¹ Isabel Sanz-Sáez,^{1,2} Clara Cardelús,¹ Isabel Ferrera,^{1,3} Josep M. Gasol,¹ Olga Sánchez⁴

AUTHOR AFFILIATIONS See affiliation list on p. 18.

ABSTRACT Isolation of microorganisms is a useful approach to gathering knowledge about their genomic properties, physiology, and ecology, in addition to allowing the characterization of novel taxa. We performed an extensive isolation effort on samples from seawater manipulation experiments that were carried out during the four astronomical seasons in a coastal site of the northwest Mediterranean to evaluate the impact of grazing, viral mortality, resource competition reduction, and light presence/absence on bacterioplankton growth. Isolates were retrieved using two growth media, and their full 16S rRNA gene was sequenced to assess their identity and calculate their culturability across seasons and experimental conditions. A total of 1,643 isolates were obtained, mainly affiliated to the classes *Gammaproteobacteria* (44%), *Alphaproteobacteria* (26%), and *Bacteroidia* (17%). Isolates pertaining to class *Gammaproteobacteria* were the most abundant in all experiments, while *Bacteroidia* were preferentially enriched in the treatments with reduced grazing. Sixty-one isolates had a similarity below 97% to cultured taxa and are thus putatively novel. Comparison of isolate sequences with 16S rRNA gene amplicon sequences from the same samples showed that the percentage of reads corresponding to isolates was 21.4% within the whole data set, with dramatic increases in the summer virus-reduced (71%) and diluted (47%) treatments. In fact, we were able to isolate the top 10 abundant taxa in several experiments and from the whole data set. We also show that top-down and bottom-up controls differentially affect taxa in terms of culturability. Our results indicate that culturing marine bacteria using agar plates can be successful in certain ecological situations.

IMPORTANCE Bottom-up and top-down controls greatly influence marine microbial community composition and dynamics, which in turn have effects on their culturability. We isolated a high amount of heterotrophic bacterial strains from experiments where seawater environmental conditions had been manipulated and found that decreasing grazing and viral pressure as well as rising nutrient availability are key factors increasing the success in culturing marine bacteria. Our data hint at factors influencing culturability and underpin bacterial cultures as a powerful way to discover new taxa.

KEYWORDS bacterial isolates, microcosm experiments, culturability

Current marine microbial ecology is largely based on culture-independent studies, yet isolation of marine microbes is still an essential process that allows performing physiological experiments and testing ecological hypotheses derived from culture-independent studies, by allowing access to whole genomes that inform about microbial metabolic capabilities and characterization of novel genes (1), through retrieval of novel taxa (2), and enabling the utilization of naturally present organisms in, e.g., bioremediation.

Editor Vincent J. Denef, University of Michigan-Ann Arbor, Ann Arbor, Michigan, USA

Address correspondence to Xavier Rey-Velasco, xavier.rey@icm.csic.es, or Olga Sánchez, olga.sanchez@uab.cat.

The authors declare no conflict of interest.

See the funding table on p. 18.

Received 28 February 2023

Accepted 3 August 2023

Published 25 September 2023

Copyright © 2023 Rey-Velasco et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

It is well known that most marine microbes have historically been recalcitrant to cultivation in a phenomenon labeled as the “great plate count anomaly” (3) or the “1% culturability paradigm” (4). This statement, however, has recently been disproved and we now know that higher-than-thought proportions of taxa have been cultured across biomes, in particular in the oceans (4–6). This is partly due to alternative culturing techniques that have been developed to increase the retrieval in culture of microorganisms from environmental samples, such as diffusion chambers (7, 8), cultivation chips (9, 10), microfluidic systems (10, 11), microencapsulation (12, 13), high-throughput culturing (14), or high-throughput dilution to extinction (HT-DTE) (15). These techniques try to overcome some of the factors that make microbes recalcitrant to cultivation, such as the requirement of specific growth factors, inorganic compounds or electron donors and acceptors which are not included in common culture media, symbiotic interdependencies which make some organisms dependent on others to be cultured, or out-competition of oligotrophs by copiotrophs (16).

Still, classic agar plates are the most economic and easy-to-implement method to culture microorganisms. This technique generally produces somehow unsatisfactory results because it is biased toward copiotrophic taxa, which normally are present in low abundances in the sea (17). However, high culturabilities have been obtained using agar plates after sudden environmentally relevant events (18), or in nutrient-rich conditions (3, 19–23), suggesting that changes in environmental conditions could lead to a larger culturing efficiency with this technique. In fact, low abundances and dormancy induced by poor nutrient availability (bottom-up control) are factors that limit the culturability of marine microbes (16). It is also known that copiotrophs are especially targeted by protists and viruses (top-down control) (24–26) and some studies propose that viral infection could be responsible for low plating efficiency (27, 28). Importantly enough, certain copiotrophs can quickly respond to changes in the environment, so that they sometimes dominate microbial communities (24, 29–31), and these environmental changes might be related to increased nutrient availability or reduced predator or viral mortality. We also know that light significantly determines seasonal changes in marine microbial communities and could thus also influence culturability (32–35).

Micro- or mesocosm experiments are a common approach to determining the effects of environmental variables on microbial abundance, activity, and diversity. They have been used, e.g., to describe the effects of phytoplankton blooms (36), oil spills (37), grazer reduction (38), or viral suppression (25) on bacterial community dynamics using molecular approaches, but so far we are not aware of culturing efforts in this type of experiments. In this study, we performed extensive bacterial isolation efforts in several manipulation experiments carried out in the four astronomical seasons that evaluated the impact of grazers, viruses, light, and resource availability on the bacterial community dynamics of the Blanes Bay Microbial Observatory (BBMO; northwest [NW] Mediterranean) using two different culture media: the standard, nutrient-rich Marine Agar 2216 (MA) and Marine Reasoner's 2A Agar (mR2A), which has lower concentration of nutrients. Our main objectives were (i) to obtain a heterotrophic bacterial collection from Blanes Bay as diverse as possible, eventually retrieving novel taxa, (ii) to test the phylogenetic compositional differences of culturable bacteria across culture media, seasons, and experiments, and (iii) to compare the isolates with 16S rRNA gene amplicon sequencing data from the same experiments to determine the influence of environmental conditions on culturability. Thus, we isolated bacteria from initial (t_0) and final times (t_f) of experiments where we manipulated seawater to remove large predators in light/dark cycles (predator-reduced light [PL]) and in the dark (predator-reduced dark [PD]), to increase nutrient availability through dilution of the original bacterial community while also reducing predators in light/dark cycles (diluted light [DL]) and to add to these manipulations virus reduction in light/dark cycles (virus-reduced light [VL]). There were also unmanipulated controls for these experiments in light/dark cycles (control light [CL]) and in the dark (control dark [CD]).

RESULTS

Composition and diversity of the isolate collection

We obtained 1,643 bacterial isolates (listed in Table S1 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx) belonging to 5 phyla, 7 classes, 24 orders, 52 families, and 125 genera that we clustered at 99% sequence similarity into 336 isolated operational taxonomic units (iOTUs) and at 100% similarity into 715 zero-radius iOTUs (ziOTUs). The number of isolates was relatively homogeneous across culture media (816 isolates in MA, 827 isolates in mR2A), seasons and treatments, and comparatively higher in t_0 samples altogether (Table 1). The most abundant isolates pertained to classes *Gammaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidia* (Fig. 1A) and genera *Alteromonas* (360 isolates), *Limimanicola* (129 isolates), *Pseudoalteromonas* (111 isolates), *Bacillus* (78 isolates), and *Alcanivorax* (71 isolates). Interestingly, two of our isolates were affiliated to the recently described class *Rhodothermia* (39) and one to *Verrucomicrobiae*.

The mean culturability, measured as the ratio of the concentration of plate colony counts (CFU mL⁻¹) and total prokaryotic cell concentration in DAPI (4',6-diamidino-2-phenylindole) samples (cells mL⁻¹), was $0.1 \pm 0.22\%$ and it almost always increased from t_0 to t_f , presenting its minimum in the fall (treatment PL at t_0 , mR2A, 0.001%) and its highest values in the fall VL t_f (the maximum was 1.4% in MA) and summer and spring DL and VL t_f (Table S2 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx).

Isolate composition and diversity across culture media

In general, culturability (CFU DAPI⁻¹) had slightly higher values in MA, with a mean of $0.11 \pm 0.24\%$ than in mR2A, with a mean of $0.085 \pm 0.2\%$ (Wilcoxon rank-sum test $P = 0.07$). The class composition of the isolates was highly similar in MA and mR2A, and principal coordinate analysis (PCoA) did not show culture media to explain any compositional variation (Fig. S1A at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). However, while 51 genera were isolated with both media, 47 were only isolated in MA and 27 were unique for mR2A (Table S3 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx). In fact, only 82 (24.4%) iOTUs were shared among MA and mR2A (Fig. 1B).

TABLE 1 Distribution of the isolates by class, season, and treatment^b

Class	Season				Treatment ^a						
	Fall	Winter	Spring	Summer	t_0	CL	CD	PL	PD	DL	VL
<i>Gammaproteobacteria</i>	42.6% (156)	45.9% (206)	29.5% (117)	58.1% (251)	33.8% (194)	39.6% (39)	35.9% (40)	55.5% (41)	52.0% (42)	56.2% (122)	56.2% (123)
<i>Alphaproteobacteria</i>	26.2% (41)	15.6% (43)	39.1% (155)	25.9% (112)	29.4% (169)	32.5% (44)	36.5% (45)	14.5% (24)	13.3% (19)	25.3% (46)	26.0% (45)
<i>Bacteroidia</i>	18.6% (47)	22.5% (48)	19.9% (49)	5.6% (23)	13.2% (50)	15.6% (23)	17.9% (27)	23.7% (51)	29.3% (52)	12.0% (25)	15.1% (32)
<i>Bacilli</i>	4.1% (53)	3.3% (53)	9.6% (37)	10.0% (54)	12.5% (55)	7.8% (12)	5.8% (9)	0.6% (1)	2.7% (4)	4.6% (10)	1.4% (3)
<i>Actinobacteria</i>	8.2% (29)	12.5% (40)	1.8% (7)	0.2% (1)	10.5% (56)	4.5% (7)	3.8% (6)	5.8% (10)	2.7% (4)	1.8% (4)	1.4% (3)
<i>Rhodothermia</i>	0.3% (1)	0.0% (0)	0.0% (0)	0.2% (1)	0.3% (2)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)
<i>Verrucomicrobiae</i>	0.0% (0)	0.2% (1)	0.0% (0)	0.0% (0)	0.2% (1)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)
Total	366	449	396	432	574	154	156	173	150	217	219

^a t_0 considers all treatments at the initial time, CL to VL correspond to the final time of each treatment.

^bRelative abundances (in %) and number of isolates in brackets are presented for each class (based on SILVA classification).

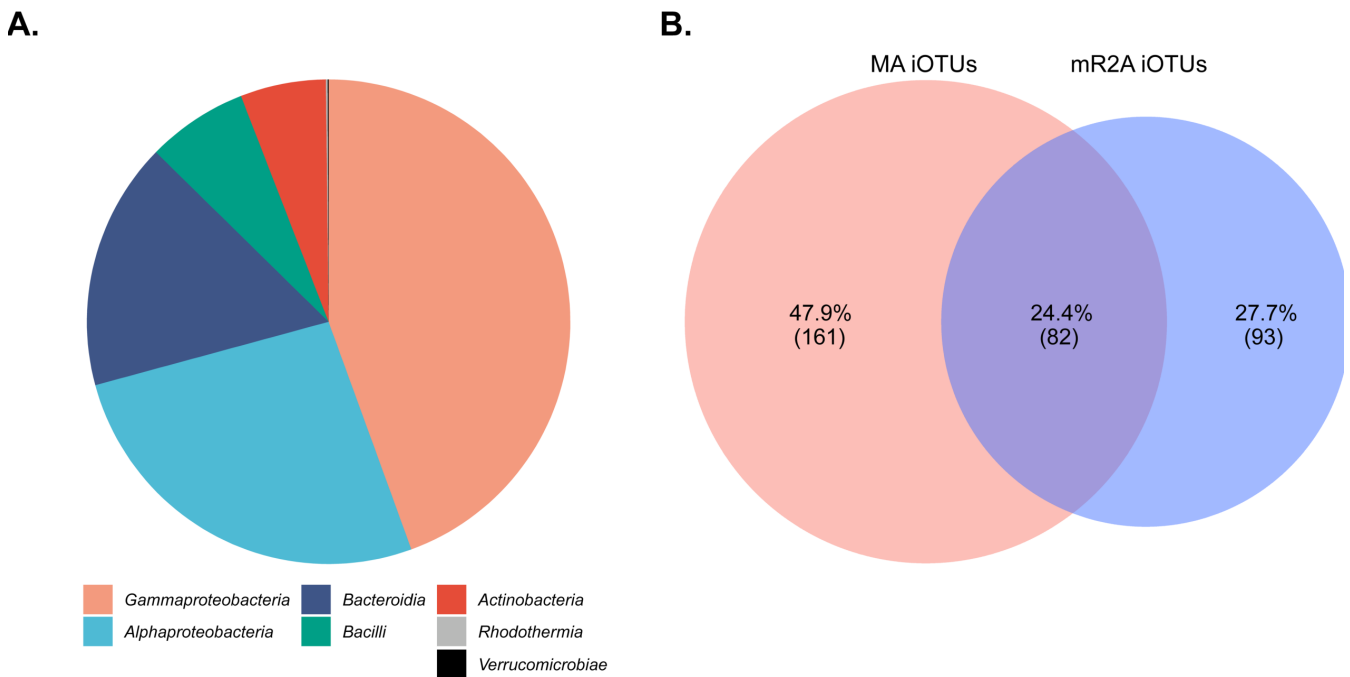


FIG 1 Overview of the isolate collection. (A) Pie chart showing proportional class distribution of the iOTUs. (B) Proportional Venn diagram showing similarity in iOTU composition between the two culture media used. Data calculated from the non-normalized iOTU table.

All α -diversity indices were significantly lower in mR2A than in MA (Fig. S1B at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). Rarefaction curves of the different media showed a similar pattern: with an equivalent sampling effort we obtained more iOTUs on MA than on mR2A and the latter was closer to reach an asymptote (Fig. S1C at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf).

Isolate composition and diversity across seasons

The compositional comparison between seasons at the class level is shown in Table 1. *Gammaproteobacteria* isolates had higher proportions in the summer experiment, while *Bacteroidia* were at their minimum. *Alphaproteobacteria* isolates were more numerous in the spring experiment, *Actinobacteria* were mostly isolated in the winter and the fall experiments, and the only isolate pertaining to class *Verrucomicrobiae* was obtained in the winter experiment.

The composition of the isolates at the iOTU level was significantly affected by season as shown in the PCoA followed by envfit analysis ($\text{Pr}[>r] < 0.001$; Fig. S2A at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). A dendrogram of the iOTU table by season using Euclidean distances indicated clustering between winter and fall, with the summer experiment clearly separated (Fig. S2B at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf).

Chao1 richness estimators and Shannon diversity indices of the isolated community were comparable in the winter and fall experiments, with lower values in the spring and especially the summer experiment. Pielou evenness and Faith's phylogenetic diversity (FPD) were lower in the summer than in the rest of the seasons and had slightly higher values in the fall (Fig. S2C at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). Rarefaction curves indicated that with a similar sampling effort (slightly lower in fall), the summer communities had almost reached the asymptote while the winter, with the highest iOTU number, was far from it (Fig. S2D at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf).

Isolate composition and diversity across treatments

To compare treatments we clustered together all t_0 samples (untreated) into one category and compared that category to each t_f (treatments). All treatments except controls were enriched in *Gammaproteobacteria* compared to t_0 . *Alphaproteobacteria* relative abundances increased slightly in the control treatments and decreased when predators were reduced. *Bacteroidia* were more abundant in predator-reduced treatments compared to the rest, while *Actinobacteria* and *Bacilli* were more abundant at t_0 (i.e., reduced their presence in all experimental treatments). The rare classes, *Rhodothermia* and *Verrucomicrobiae*, were both isolated only in t_0 samples. On the other hand, light did not show any notable influence in isolate class composition (Table 1).

Regarding isolate similarity, the PCoA followed by *envfit* analysis showed that treatments explained the variance of iOTU composition with a significant goodness of fit ($P_{[>r]} < 0.001$) with t_0 samples opposed to PL, PD, DL, and VL treatments, with t_f controls half the way between t_0 and treatments t_f (Fig. S3A at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf).

Due to the high variance of the t_0 samples and the similarity of the light and dark treatments (Table 1), we only tested the α -diversity indices at t_f of the light treatments. In general, isolates in DL and VL had the lowest values of the Chao1 estimator, Shannon diversity, Pielou evenness, and FPD indices while CL and PL had similarly higher values (Fig. S3B at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). ANOVA and Tukey's post-hoc test showed no significant differences between these values, probably due to the low number of samples in each category. Rarefaction curves (Fig. S3C and D at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf) concur with this observation: at final times, DL and VL were the treatments with the lowest number of iOTUs despite being the most sampled.

Comparison between isolate diversity and amplicon 16S rRNA gene diversity

We compared the complete 16S rRNA gene sequences of our isolates with the V4-V5 region of the same gene in the amplicon sequence variants (ASVs) from the same experiments, and we found that 63.08% of our zIOTUs matched to an ASV with 100% similarity. We isolated 173 out of 4,594 ASVs (3.76%) accounting for 21.37% of the reads in the whole data set, with high variability between samples: there were lower values in t_0 samples with a mean of $2.44 \pm 2.78\%$ than at t_f , with a mean of $9.97 \pm 16.38\%$ (Wilcoxon rank-sum test $P < 0.01$). Importantly, this value escalated in summer and spring VL and DL treatments (Fig. 2; Table S4 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx displays a complete list), reaching as high as 70.76% cultured reads in the summer VL treatment and 47.09% in spring VL. It also increased notably in the summer CD treatment, while in the fall and winter VL treatment the values were more modest but still higher than at t_0 .

While in most samples we cultured taxa pertaining to the rare biosphere (Fig. 3A; Fig. S4 at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf), in some we isolated very relevant ASVs in terms of abundance (Fig. 3B). We cultivated organisms that were 100% equal to almost all the top rank taxa detected by amplicon sequencing of the V4-5 region of 16S rRNA gene in the summer experiment VL and DL t_f treatments, affiliated to genera *Alteromonas*, *Vibrio*, and *Limimariicola*. Interestingly, we also isolated ranks six and eight of summer CD treatment at t_f that pertained again to genus *Alteromonas*. In the spring experiment, we cultured the first ASV of VL treatment t_f and the second from DL treatment t_f , affiliating to genus *Nereida*. In the fall and winter experiments, we obtained more modest but still notable results from treatment VL t_f . Ranks four, five, and eight (genera *Alteromonas*, *Halomonas*, and *Nereida*) were isolated in the fall experiment while in the winter experiment we cultured organisms 100% identical to ranks seven, eight, nine, and ten (genera *Vibrio*, *Polaribacter*, *Lentibacter*, and *Colwellia*). We isolated *Pseudoalteromonas*, which was in rank eight of winter DL t_f , and *Tenacibaculum*, which appeared in important positions at t_f of the winter, fall, and spring VL

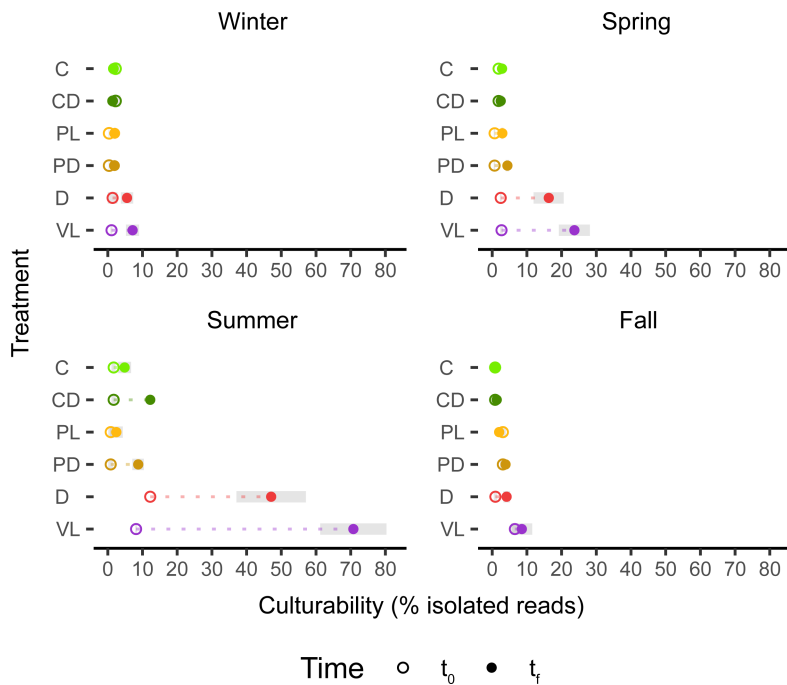


FIG 2 Cultured proportion of the population in each season, treatment, and time. Percentage of Illumina 16S rRNA gene reads that corresponds to isolates at 100% identity vs treatments. Hollow circles represent t_0 , full circles represent t_f . Gray areas indicate standard deviations of replicates.

experiments (ranks 14, 16, and 19, respectively). Surprisingly, there were some t_0 samples from where some isolates were identical to relevant taxa: the summer experiment, treatment DL t_0 (ranks three and four, *Limimanicola* and *Palleronia*), and the fall experiment VL t_0 (rank four, *Halomonas*). Importantly, we isolated organisms 100% identical to ranks three, six, seven, and nine of the whole data set pertaining to genera *Alteromonas* and *Vibrio*. Ranks, mean abundances, and closest neighbors of all the ASVs with identical cultured organisms can be found in Table S5 (at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx) for each season and treatment and Table S6 (at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx) for the data set as a whole.

To decipher the link between culturability and the manipulations done in this study, we integrated the information of *in situ* ASV relative abundances (Table S5 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx) and the frequency of isolation for the most relevant genera (those which reached high abundances in the treatments, and the 10 most cultured) in Fig. 4. This shows that especially *Alteromonas* were very frequently cultured in all the t_f of the experiments, coupled with an increase in their relative abundances. A similar but slightly less strong trend could be seen for *Pseudoalteromonas*. Other genera that were more culturable and abundant in some or all of the manipulations were *Limimanicola*, *Nereida*, *Vibrio*, *Tenacibaculum*, *Dokdonia*, *Polaribacter*, *Colwellia*, and *Lentibacter*. All of them showed higher culturabilities in specific treatments, for example, *Limimanicola* were more culturable in the control, DL, and VL treatments, which correlated with their abundances; *Vibrio* were more culturable in DL while in VL, despite being more abundant, were not cultured; *Dokdonia* preferred predator-reduced treatments despite being more abundant in the stronger manipulations (DL and VL) and *Colwellia* were only cultured in some of the manipulations. On the contrary, *Palleronia*, *Erythrobacter*, *Halomonas*, *Bacillus*, and *Alcanivorax* were more culturable in t_0 samples, despite sometimes being more abundant at the end of the manipulations.

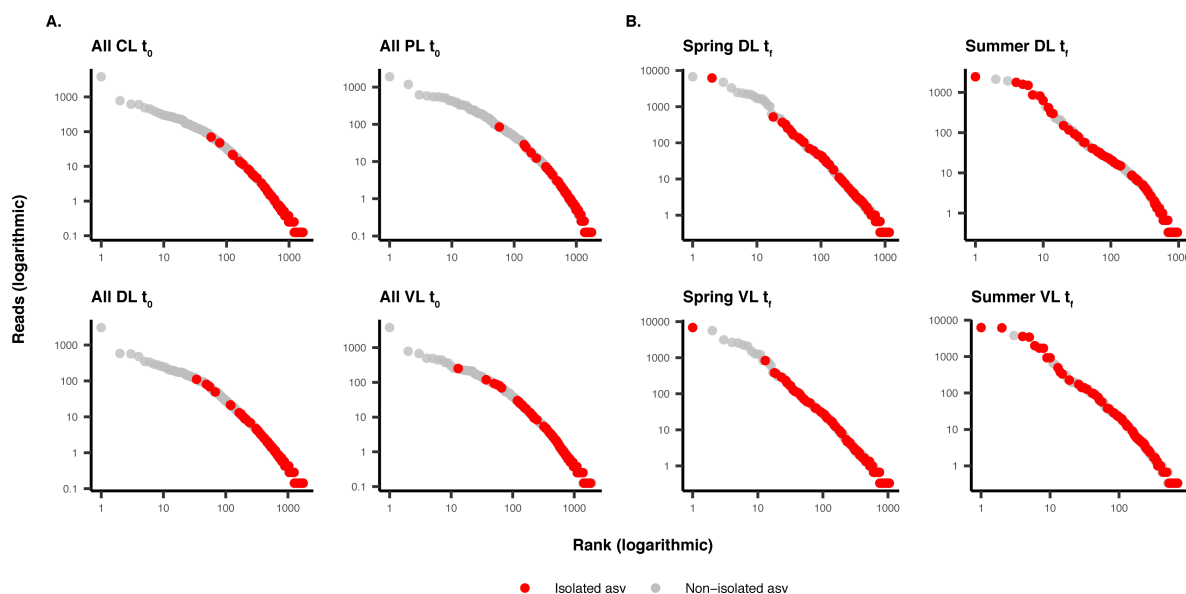


FIG 3 Selected rank abundance plots based on 16S rRNA gene amplicon sequencing (region V4-V5) with indication of the cultured organisms that match the sequences (in red). The non-isolated ASVs are represented in gray. (A) Examples where we only isolated rare taxa. All seasons are considered. (B) Summer and spring samples where we isolated dominant taxa.

Novelty of the isolate collection

To test the novelty of our collection, we plotted the closest environmental match (CEM) vs the closest cultured match (CCM) of our isolates (Fig. 5). This presents 61 isolates (3.7% of total isolates) that belong to 39 iOTUs (11.6% of total iOTUs) and 48 ziOTUs (6.7% of total ziOTUs) with less than 97% similarity with their CCM (i.e., they correspond to taxa that have never been cultured). Thirty isolates (1.8%) scored between 94.5% and 97% similarity with both CCM and CEM, which could represent 19 genuinely novel species, as was revealed by clustering them to 97% similarity. Moreover, two isolates had less than 94.5% similarity with neither their CCM nor CEM, and thus they could represent two novel genera (40).

To examine the phylogenetic placement of the novel strains, we constructed a phylogenetic tree with the putative novel ziOTUs (Fig. 6), which indicates that novel isolates pertained mostly to classes *Bacteroidia*, *Gammaproteobacteria*, and *Alphaproteobacteria*. There were three ziOTUs affiliated to class *Bacilli*, and two ziOTUs to class *Rhodothermia*, which interestingly were the only ones from this class in the whole collection. Novel isolates were mostly cultured in MA (35 ziOTUs in MA, 13 in mR2A) and distributed similarly between seasons. Most novel ziOTUs were obtained from t_0 samples (twenty-six) with some in control treatments (eleven), predator-reduced (nine), and less frequently DL (four) and VL (one) treatments.

DISCUSSION

We have obtained an extensive collection of 1,643 isolates from different manipulation experiments that were carried out in the four astronomical seasons, using two distinct culture media. Overall, the phylogenetic distribution of our collection at the class level (Table 1) is similar to that obtained in other studies made in the same sampling site (41). It is noteworthy, though, that classes *Rhodothermia* and *Verrucomicrobiae* had never been isolated in the BBMO, though the latter had been detected by culture-independent approaches (e.g., reference 41). In addition to this general picture, one of the aims of this study was to test differences in the phylogenetic composition and diversity of our isolates across culture media, seasons, and treatments.

Culture medium MA has a higher organic matter concentration (5 g L⁻¹ proteose peptone, 1 g L⁻¹ yeast extract, total 6 g L⁻¹ organic matter) than mR2A, while the latter

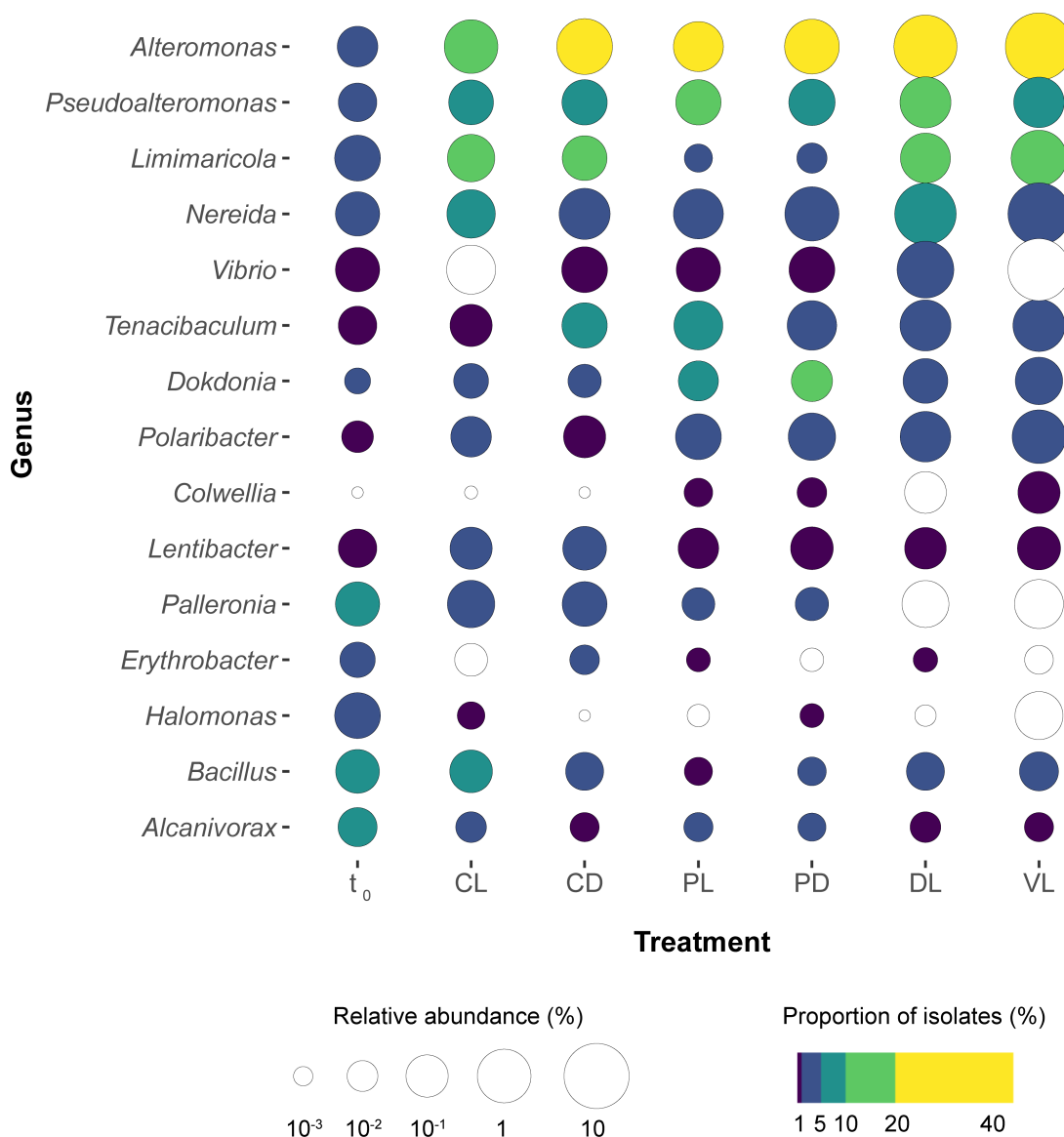


FIG 4 Culturability (proportion of isolates) and relative abundances of relevant genera in this study in the different treatments. White color means that the particular genus was not isolated in that treatment. Category t₀ incorporates all treatments at the initial time; CL to VL correspond to the final times of each treatment.

presents a more diverse composition in terms of carbon sources (0.5 g L⁻¹ proteose peptone, 0.5 g L⁻¹ casamino acids, 0.5 g L⁻¹ yeast extract, 0.5 g L⁻¹ dextrose, total 2.5 g L⁻¹ organic matter); therefore, the use of these two media aimed to increase the diversity of the obtained heterotrophic isolates. This goal was achieved, as the proportion of shared genera (Table S3 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx) and iOTUs (Fig. 1B) across culture media was low. We expected to obtain higher CFU mL⁻¹ in mR2A considering that previous studies have shown that more oligotrophic culture media resulted in better culturabilities than standard rich media (14, 42). However, in this study mR2A yielded slightly lower values than MA. This could have happened because mR2A is not oligotrophic (i.e., low-nutrient) enough to manifest this effect, and after all it still contains 2.5 g L⁻¹ organic matter. Probably, using a more nutrient-poor medium such as the modified seawater medium (SW) (14, 43) could have led to the isolation of other taxonomic groups adapted to the low nutrient availability observed in the environment.

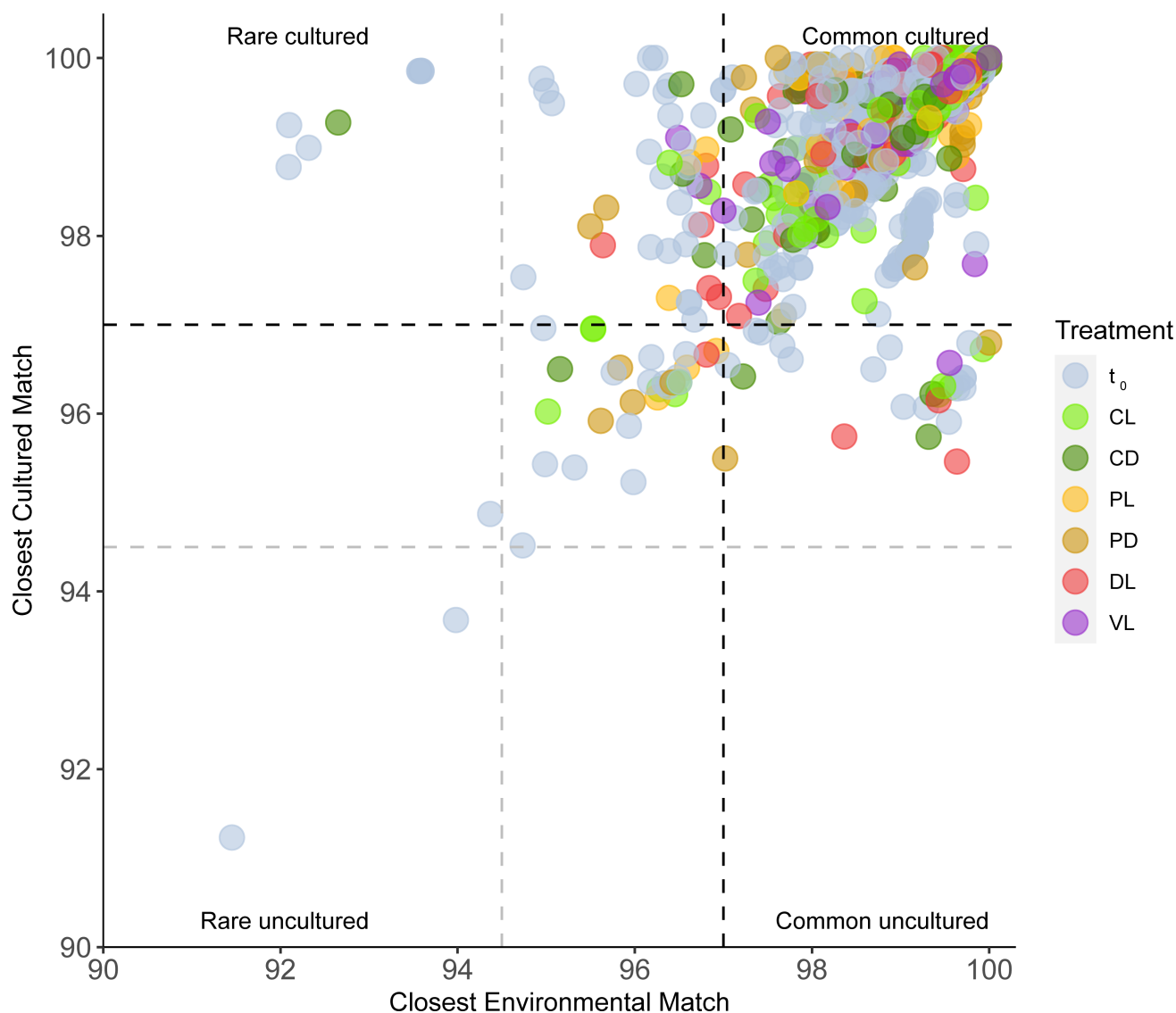


FIG 5 Percentage similarity between the CCM and the CEM of isolate 16S rRNA gene sequences. Horizontal and vertical lines represent the typical cut-off values of 97% (black dashed lines) commonly used for species delineation, and the cut-off values of 94.5% (gray dashed lines) used for genera delineation. Colored by treatment. Category t_0 incorporates all treatments at the initial time; CL to VL correspond to the final times of each treatment.

The isolate composition varied significantly across seasons: fall and winter were the most similar seasons and summer the most distant (Fig. S2B at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). This is the exact same trend that was observed for the structure of the environmental bacterial communities in the BBMO as determined by DGGE in Alonso-Sáez et al. (34), and by 16S rRNA gene amplicon sequencing (44). Our isolates reached their lowest α -diversity in summer with higher values in the spring and, especially, in the fall and winter (Fig. S2C at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). Other studies carried out in the BBMO using molecular methodologies have shown similar tendencies (34, 44). While the seasonality of microbial communities in BBMO has been broadly described (32, 34, 44–46), this is the first evidence of seasonality in the culturable bacteria fraction of the BBMO community.

The dominance of Gammaproteobacterial isolates in all treatments except the controls (Table 1) correlates with CARD-FISH relative abundances in these experiments (32) which were seen to be high for this class and especially for *Alteromonadaceae* in the

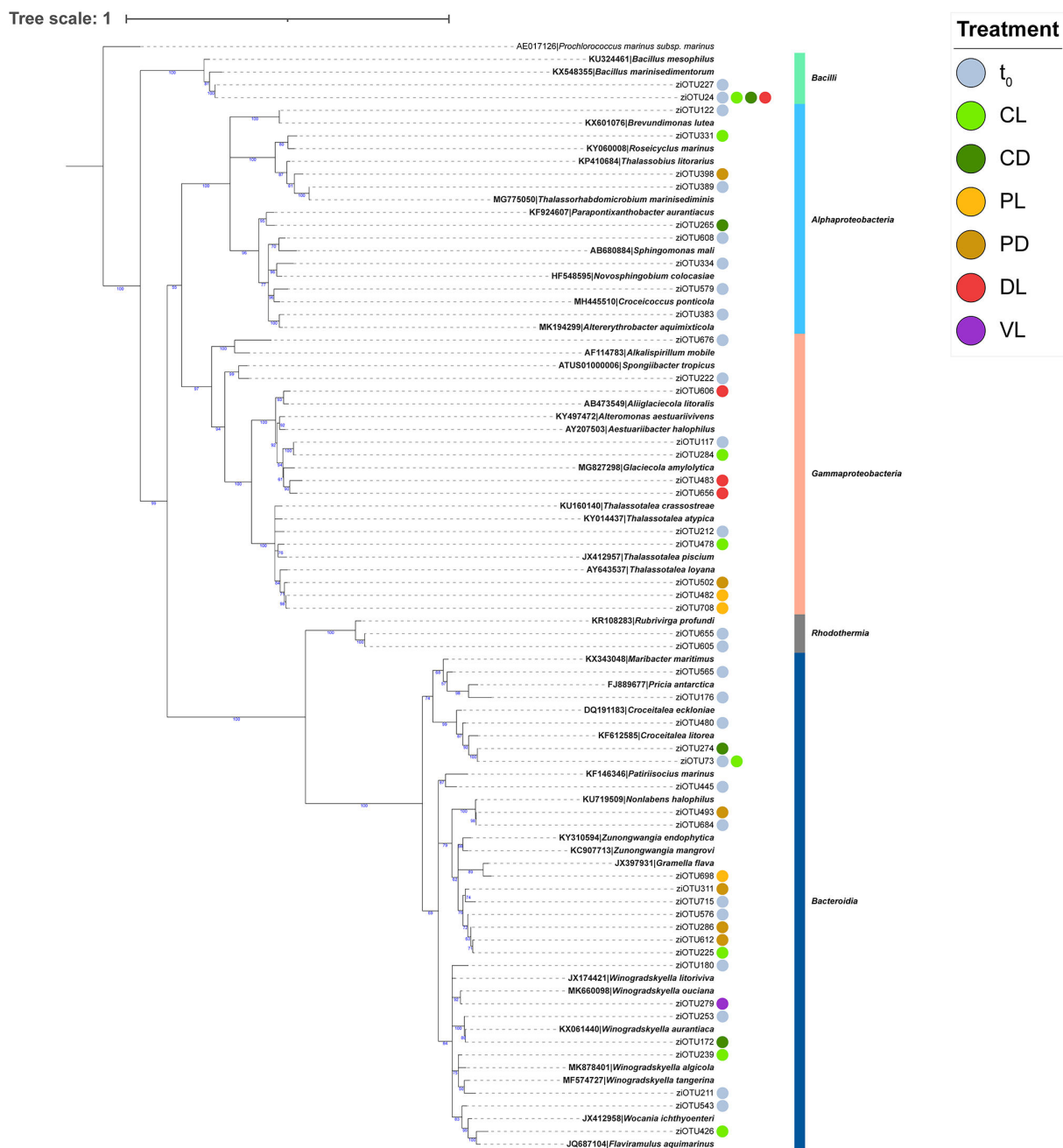


FIG 6 Phylogeny of putative novel isolates. Phylogenetic tree includes 48 zOTUs with <97% similarity to their CCM in the RDP database and their closest match in the SILVA Living Tree Project (in bold). The numbers in the nodes represent bootstrap coefficients calculated from 1,450 replicates. Non-supported branches (bootstrap coefficients below 50%) were collapsed. Colored circles represent the treatments from where the zOTUs were isolated. Classes to which the different taxa pertain are also indicated. Category t₀ incorporates all treatments at the initial time; CL to VL correspond to the final times of each treatment.

mentioned samples. Likewise, the isolation of comparatively more taxa affiliating to class *Bacteroidia* in the PL and PD treatments (Table 1) is in accordance with the same data: CARD-FISH abundance corresponding to this class peaked especially in the winter and spring PL and PD treatments (32). The PCoA by treatment (Fig. S3A at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf) suggests that while the sole fact of confining seawater in bottles caused a change in the composition of culturable bacteria, a known effect that has already been reported (47, 48), treatments that implied a real manipulation of environmental conditions (PL, PD, DL, and VL) had by themselves a notable effect in changing the composition of the culturable bacterial

community. This compositional change did not affect α -diversity in PL and PD; however, it had an effect in DL and VL treatments (Fig. S3B at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf), where α -diversity decreased, suggesting that these treatments favored a narrow set of culturable bacteria over the other treatments. In the microcosm experiments performed by Teira et al. (38), the α -diversity indices were reduced when reducing grazer pressure (equivalent to the PL treatment here), but here it did so only with deeper manipulations (DL and VL). While Teira et al. performed their experiments in offshore waters in different oceans (Atlantic, Pacific, and Indian) that were analyzed with 16S rRNA gene amplicon sequencing, we observed a similar trend in the coastal Mediterranean sea.

When comparing the 16S rRNA gene sequences of isolates and ASVs (V4-V5 region), we observed that 63.08% of our zOTUs were identical to an ASV and 3.76% of all ASVs were represented by isolates. It is common to find similar or lower proportions of isolates represented by sequences detected using molecular approaches (14, 22, 41, 49, 50). This is because taxa recovered by isolation usually belong to the “rare biosphere” of a given environment, while molecular techniques retrieve preferentially the relatively more abundant bacteria (17, 51). The low proportion of ASVs represented by isolates is actually similar to the value found in other studies (23, 50).

In this study we demonstrate that, using agar plates, it is possible to isolate dominant heterotrophic marine bacteria that represent a significant proportion of the population. In fact, our isolates accounted for 21.37% of the total reads and this percentage was higher in certain samples, reaching up to 70.76% (in the summer experiment, treatment VL, Fig. 2; Table S4 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx). Studies that compare isolates with sequencing data are scarce and they usually do not provide culturability values. Among the ones that made available these data, Sanz-Sáez et al. (22) obtained a mean of 0.3–1.1% with 7.8% as the maximum proportion of reads matching with isolates in samples from global oceans at different depths. Wang et al. (23) isolated 45% of their whole 3-sample data set from marine sediments, yet they applied a 97% similarity threshold to consider an OTU as cultured and thus, this number was likely an overestimation in comparison with our approach. Alejandro-Colomo et al. (14) obtained a mean of $5.75 \pm 2.99\%$ cultured reads during a phytoplankton bloom in the North Sea, with a maximum of 11.51% in one sample (calculated from their Table S4 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx); however, they could not isolate any of the most abundant taxa. Sanz-Sáez et al. (52) obtained isolates representing up to 45% of the reads in the bathypelagic particle-associated fraction and isolated the most abundant ASVs in this fraction and ocean depth, reinforcing the idea that isolation of heterotrophic bacteria can be successful in certain environments or ecological situations.

Other studies that used alternative culturing techniques in natural samples, mainly HT-DTE, have obtained similar results in terms of the proportion of isolates matching to sequences seen through culture-independent approaches, but much better results in terms of culturability. While we isolated 3.76% of the total ASVs, Henson et al. (15) obtained 5% of them in coastal samples, although they used a 99% identity threshold. Bartelme et al. (53) obtained 20% culturability from soil samples in their HT-DTE experiments, with 11% of the total community isolated at 97% identity, while Yang et al. (54) and Connon et al. (55) obtained, respectively, 12% and 14% culturability in surface waters. Interestingly, Kim et al. (56) reached up to 34.6% culturability in a freshwater sample with a modified HT-DTE protocol supplemented with catalase. Other studies have isolated top-abundant taxa with flow sorting combined with diverse culture media in soil samples (57) or a combination of diffusion chambers and HT-DTE in coastal waters (58). All the above-mentioned studies obtained higher culturability values compared to our study, in which we reached a maximum of 1.4% (Table S2 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx). Thus, it seems that techniques designed for culturing oligotrophs are best to isolate the microbial majority in non-experimentally modified seawater conditions.

In general, the DL and VL treatments were the ones with the highest proportions of reads in the 16S rRNA gene amplicon sequencing data set matching exactly with an isolate (Fig. 2). The ASVs that accounted for high percentages of reads in these treatments and were 100% identical to isolates in this collection (Tables S5 and S6 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx) mostly pertained to genera *Alteromonas*, *Vibrio*, *Limimarinicola*, and *Nereida*, fast-growing r-strategists that have commonly also been isolated in other studies (14, 22, 41), and affiliate to phylogenetic groups which have been reported to actively respond to the increase of resource availability in the environment during events like phytoplankton blooms (30, 36, 59–61). In this case, treatments DL and VL provided increased resource availability. In view of these data, it looks like the high proportions of reads affiliating to cultured taxa in this study are caused by the effects of the experimental manipulations, especially the increase in nutrient availability in DL and VL, that favored the growth of copiotrophic taxa which are normally found in the environment as part of the rare biosphere (17) but can become dominant under certain conditions, such as the ones created in the experiments (25, 31, 62, 63). This also suggests that r-strategists are more strongly limited by bottom-up (resources) controls than by top-down (grazers and viruses) factors.

The effect of experimental treatments in terms of isolation success (i.e., having cultured top-rank taxa and high percentages of reads affiliating to isolates) appears to be markedly different between seasons. Summer was clearly the most successful season, followed by spring, while in the fall and winter experiments, the various treatments had very weak effects (Fig. 2; Fig. S4 at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf). This could partially be explained by looking at the identities of the most abundant ASVs that we did not isolate in this study. In the DL and VL treatments (t_f), the most abundant uncultured taxa accounting for a high proportion of fall, winter, and spring reads affiliated to a total of 12 species in the NCBI 16S rRNA database (Table S7 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx) and, except for *Donghicola eburneus*, they had been isolated in different conditions (culture media or temperature) and/or from other environments different than the ones used in this study (see references in Table S7 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx). It is also important to note that *in situ* temperatures in summer (21.3°C [32]) and fall (19.5°C [32]) were the closest to the one used in this study (RT; 20–25°C). All this suggests that isolation success was higher in summer and spring because dominant taxa in these seasons were easier to culture with our methods, especially our used culture media.

We isolated low proportions of the community in t_0 samples (Fig. 2) and, except for some specific treatments and seasons, we did not isolate dominant taxa in terms of abundance (Fig. 3A). Most of the top-abundant ASVs in untreated samples pertain to oligotrophic genera such as *Prochlorococcus* or *Pelagibacter* that cannot be isolated in agar plates using nutrient-rich media. On the contrary, they have been isolated with methods such as HT-DTE (15) or the use of complex media (64). A taxon affiliating to species *Aquibacter zeaxanthinifaciens* was among the top ASVs but, in contrast to this study, had been isolated using the DTE plating method (65). This supports the idea that a change in the microbial community caused by the manipulations selected more readily culturable microorganisms (at the final times), so taxa that are normally abundant in the environment (the ones in initial times) are missed by our approach.

It is well known that PCR-based sequencing overrepresents the abundances of bacteria with multiple 16S rRNA gene copies (66). In fact, all our isolates that accounted for high numbers of reads are known to harbor numerous copies of the ribosomal operon according to the rrnDB database (67); therefore, it is likely that our proportions of reads corresponding to isolates are overestimated. For this reason, we generated a revised ASV abundance table by dividing each ASV read value by the number of 16S rRNA gene copies obtained from rrnDB (67) according to their class assignment, and recalculated the proportions of cultured reads in relation to these “corrected” values.

With “correction,” the mean proportions of reads corresponding to isolates changed from $6.20 \pm 12.23\%$ to $5.48 \pm 10.70\%$ and the raw maximum of 70.76% decreased to 59.66% (Table S4 at https://github.com/x-rv/Manuscript-2023/raw/main/Supplemental_Tables.xlsx). These estimations would only slightly reduce our isolation success, showing nonetheless the same trend.

We wanted to explore how the different treatments influenced the culturability of genera that were relevant in this study, also taking into account their abundances as determined by amplicon sequencing. We know that when using rich culture media, the culturability of a given taxon is determined by its ability to grow faster than potential competitors (16). Thus, the organisms with increased culturability in a given experiment would be the most adapted to grow on the solid media used and the particular experimental conditions. It is shown in Fig. 4 that the genus most benefited by the manipulations in terms of culturability was *Alteromonas*, which developed better than all the other taxa even in the control treatments, in accordance with its well-known opportunistic behavior (47). The culturability of *Pseudoalteromonas* also increased in all manipulations, but its higher isolation in PL and DL indicates that this genus might be limited both by predators and nutrient availability, and that light could be beneficial for its cultivation. Some species of *Pseudoalteromonas* are photoheterotrophs and, in fact, they have been seen to have higher abundances in the light compared to darkness (68). *Limimanicola* seems to be an opportunistic genus scarcely influenced by grazing, given that its abundance and culturability did not increase in the PL and PD treatments. The comparably higher cultivation of genera *Nereida* and *Vibrio* in the DL treatment suggests that they are especially subject to nutrient availability. Importantly, *Vibrio* was not isolated in the VL treatment despite being more abundant than in the rest of the experiments, indicating that the presence of virus might favor its growth on solid media, or that other groups are more sensitive to viral lysis. *Tenacibaculum* and *Dokdonia* were clearly benefited by predator reduction while *Polaribacter* seems to develop well in all conditions. The genus *Colwellia* was only isolated in the manipulations, where it also increased in relative abundance, suggesting poor cultivation in unmanipulated conditions and a high influence of predation and viral lysis. The slight increase of *Lentibacter* isolation in controls implies that this genus has an opportunistic behavior, but it does not develop well on plates when manipulations are stronger. Finally, genera *Palleronia*, *Erythrobacter*, *Halomonas*, *Bacillus*, and *Alcanivorax* were more easily isolated in initial times despite sometimes being more abundant in the manipulations, suggesting that they are less adapted to grow on plates than other taxa when conditions are favorable to copiotrophic growth. Copiotrophs are known to be especially subject to grazer control, influenced by nutrient availability and targeted by viruses (24–26); however, we have shown here that each taxonomic group seem to be differentially influenced by these factors in terms of culturability (using agar plates) and how some genera are more competitive (i.e., in terms of developing on the plates) than others when limitations are reduced.

The other main outcome of this study is that our isolation effort resulted in a high proportion (3.7% of all isolates) of putative novel taxa (Fig. 5). This novelty is comparatively higher than that found in other studies focused on isolation, such as the one from Ma et al. (69), in which 1.9% isolates represented potential novel species in samples from deep-sea water and sediments from the Mariana Trench using six culture media, or the 0.2% isolates representing novel genera in samples from different depths across the global ocean plankton using one culture medium (22). This could be explained as a result of the extensive isolation carried out from a single sample from one site and also by the colony-picking strategy seeking different morphologies. It is noteworthy that the rarefaction curve of the study (Fig. S1D at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf) had not reached an asymptote, implying that a greater isolation effort could have resulted in the discovery of more novel isolates. In fact, higher numbers of potential novel isolates have been obtained with a remarkable culturing effort using high-throughput isolation

techniques (14). The novelty obtained in this study was not equally relevant across culture media as more novel zOTUs were found in MA, which is logical if we attend to the alpha-diversity values (Fig. S1B at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf) and rarefaction curves (Fig. S1C at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf): MA was less selective than mR2A and permitted the culturability of a wider range of bacteria. It is also noteworthy that most putative novel taxa were isolated from t_0 and control treatments. Taking into account that the sampling effort at t_0 (considering all samples) was 3–4 times higher than that of the treatments at t_f (Table 1), it is not surprising to find more novel taxa there. Also, it is coherent that the frequency of isolation of novel taxa diminishes as the manipulation of environmental conditions is stronger, since it implies the selection of a smaller set of taxa. Alpha-diversity (Fig. S3B at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf) and rarefaction curves (Fig. S3C and D at https://github.com/x-rv/Manuscript-2023/blob/main/Supplemental_Figures.pdf) concur with this idea: among final times, less diversity was found in the more manipulated treatments (DL and VL).

We conclude that our extensive isolation effort applied to experimental manipulation experiments resulted in the isolation of responsive taxa corresponding to exceptionally high proportions of the microbial population, although they were mainly common copiotrophs. We are far from being able to obtain all the natural environment microorganisms in culture, but our data show that we can find a reasonable number of the bacteria responding to manipulations, indicating that culturability is highly influenced by environmental factors, especially resource availability, grazing, and viral lysis.

Overall, our results point to environmental conditions as key factors influencing isolation success. Also, culturing microorganisms with traditional methods proves useful to discover novel taxa and isolate those members of the community that are most abundant under certain environmental conditions.

MATERIALS AND METHODS

Origin of samples

Surface seawater samples were collected from the BBMO in the NW Mediterranean (41°40'N, 2°48'E), about 70 km north of Barcelona, and approximately 1 km offshore. Samples were collected on the four astronomical seasons: winter (21 February 2017), spring (26 April 2017), summer (5 July 2017), and fall (7 November 2017) and water was filtered *in situ* through a 200- μ m mesh and transported to the laboratory within 2 h.

Manipulation experiments

Six experimental treatments were set up the following day for each season as described in Sánchez et al. (32). Briefly, the treatments consisted of the following: (i) unfiltered seawater in light/dark cycles (CL) and in the dark (CD), (ii) seawater prefiltered through a 1- μ m filter to remove large predators while preserving most bacteria in light/dark cycles (PL) and in the dark (PD), (iii) unfiltered seawater diluted 1/4 with 0.2- μ m-filtered seawater to reduce predators and increase nutrient availability for bacteria in light/dark cycles (DL), and (iv) unfiltered seawater diluted 1/4 with 30-kDa-filtered seawater to reduce predators, viruses, and to increase nutrient availability, in light/dark cycles (VL). The different treatments were incubated in triplicated 9 L Nalgene bottles for 48 h at *in situ* temperature (see Table 1 in Sánchez et al. [32]) in a water bath with circulating seawater. Light treatments were limited to photosynthetically active radiation, and dark treatments were covered with several layers of dark plastic.

Samples were taken for bacterial isolation at the same time as samples for inorganic nutrient concentration and other ancillary data (reported in reference 32), DAPI total counts, CARD-FISH, and 16S rRNA gene amplicon sequencing. Samples were obtained at times 0 h, 12 h, 24 h, and 36 h in summer and winter or 48 h in the fall and spring

experiments. For isolation, 1 mL seawater subsamples were mixed with 75 μ L dimethyl sulfoxide (DMSO) in cryovials that were stored at -80°C in triplicates. In the winter experiment, samples for isolation were not taken at 0 h; therefore, 12 h after the start of the experiments was our initial time for isolation in this season.

Community DNA extraction and sequencing

For 16S rRNA gene amplicon sequencing, samples were prefiltered through a 20- μ m mesh to remove large particles, and microbial biomass was concentrated onto 0.2- μ m polycarbonate filters using a peristaltic pump. About 2–4 L were filtered from each replicate of all treatments. We extracted the DNA from the filters as described in Massana et al. (70), and then purified and concentrated it using Amicon 100 columns (Millipore) and quantified it in a NanoDrop-1000 spectrophotometer (Thermo Scientific). We stored the DNA at -80°C and an aliquot from each sample was used for sequencing using a MiSeq sequencer (2 \times 250 bp, Illumina). A first run was sent to the Integrated Microbiome Resource (Halifax, NS, Canada; <https://imr.bio>) and a second run was sent to the Research and Testing Laboratory (Lubbock, TX, USA; <http://rtlgenomics.com/>) in order to improve the quality of some of the samples. Primers 515F-Y (5'-GTG YCAG CMG CCG CGG TAA) and 926R (5'-CCG YCA ATT YMT TTR AGT TT) from Parada et al. (71) were used to amplify the V4-V5 regions of the 16S rRNA gene.

Amplicon sequencing data processing and taxonomic classification

We obtained two different runs of sequences that needed to be processed separately. Initially, we used cutadapt (72) to trim primers. Then, ASVs were obtained running DADA2 1.18.0 version (73), which consisted of different steps. The qscore plots were used to inspect the quality of our samples and decide where to trim. The subsequent step was running the DADA2 process using the pool method to increase sensitivity to sequences that might be present at very low frequencies in multiple samples. Finally, we merged the two runs and removed chimeras keeping $82.7\% \pm 0.1$ sequences per sample and obtaining the final ASV table. Taxonomic assignment was performed with DECIPHER 2.16.1 version (74) at 60% confidence aligning against the SILVA database (SILVA_SSU_r138_2019.RData). Four samples with <5,000 reads were discarded, keeping a total of 308 samples for further analyses.

Isolation of bacteria

Initial experiment sampling times (t_0) and final (t_f) of dark/light treatments from each season were used for isolation. Samples from 0 h of dark treatments from the control and predator-reduced experiments were not employed for this purpose, since at that point there had not been time for the light regime to cause an effect; therefore, the total number of samples was 42. Two different solid culture media were used, MA (Difco) and mR2A, which consisted of R2A Agar (Difco) prepared in Milli-Q water with 40 g L^{-1} Sea Salts (Sigma) (75), pH adjusted to 7.6. For liquid cultures, Marine Broth 2216 (Difco) and mR2A Broth prepared with R2A Broth (Neogen) in Milli-Q water with 40 g L^{-1} Sea Salts (Sigma) were used.

Aliquots of 100 μ L of undiluted, 1:10, and 1:100 diluted seawater were spread in triplicates on agar plates and incubated at room temperature (20–25 $^{\circ}\text{C}$) until no more colonies appeared (maximum 30 days). Colonies with different morphologies were selected from each sample and streaked on new agar plates in order to obtain pure cultures. These cultures were then transferred to liquid medium, and after turbidity was detected, 100 μ L from the suspensions were kept at -20°C for DNA extraction, while the rest was stored in 25% glycerol in cryovials at -80°C . Culturability was calculated as the ratio between the concentration of CFU mL^{-1} in agar plates and the total concentration of cells obtained from DAPI counts (DAPI mL^{-1} , data obtained from Sánchez et al. [32]).

PCR amplification and sequencing of isolates

Genomic DNA was extracted from 100 μ L of liquid cultures incubated 10 min at 99°C in a thermal cycler and 10 min at –20°C for three times. These extractions were used to PCR-amplify the nearly complete 16S rRNA gene with primers 27Fmod (5'-AGR GTT TGA TCM TGG CTC AG-3') and 1492Rmod (5'-TAC GGY TAC CTT GTT AYG ACT T-3') from Page et al. (76). Each PCR reaction was composed of 32.75 μ L Milli-Q water, 10 μ L 5 \times Green GoTaq Reaction Buffer (Promega), 1 μ L dNTPs mix (each deoxynucleotide at 10 mM), 2 μ L of each primer (10 μ M), 0.25 μ L GoTaq DNA Polymerase (Promega), and 2 μ L of extracted DNA. When faint or no bands were observed on agarose gel electrophoresis following PCR, DNA extraction was repeated with DNeasy Blood&Tissue Kit (Qiagen) following the manufacturer's recommendations. Purification and OneShot Sanger sequencing of PCR products was carried out by Genoscreen (Lille, France) with the two above-mentioned primers.

Isolates data processing and taxonomic classification

The sequences were manually quality-checked, trimmed, and assembled with Geneious software v.2022.0.1 (77). The UCLUST algorithm from USEARCH software (78) was used to cluster sequences at 99% similarity (79) in order to infer iOTUs. These iOTUs were used for all the subsequent analyses except to compare isolates and ASV sequences and to compute a phylogenetic tree to describe putative novel isolates, for which a 100% identity clustering was performed to define ziOTUs. Taxonomic classification was performed with the SINA aligner (80) against SILVA (release 138.1) (81), RDP (release 11) (82), and GTDB (release 202) (83). Additionally, isolates sequences were submitted to BLASTn v.2.12.0+ (84) against a subset of the RDP database containing only cultured taxa (CCM) and another one containing only uncultured taxa (CEM) in order to test for their novelty. All ziOTUs that had less than 97% similarity with their CCM were considered as putatively novel strains. To assess the number of putative novel species and genera, the USEARCH software was used to cluster putative novel isolates to 97% and 94.5% similarity.

Phylogenetic analyses

Two phylogenetic trees were constructed, one with all iOTUs to assess diversity of the complete collection, and another one only with putative novel ziOTUs to examine the phylogenetic placement of the novel strains. The general iOTUs tree was computed without reference sequences since it was only used to calculate phylogenetic diversity. For the tree with putative novel isolates, the closest sequence in SILVA Living Tree Project (LTP_12_2021) (81) for each ziOTU was inferred with BLASTn (84) and *Prochlorococcus marinus* subsp. *marinus* (ref AE017126 from LTP_12_2021) used as an outgroup. In both cases, the sequences were aligned with ClustalW in Geneious software v.2022.0.1 (77) and unaligned ends were trimmed. Phylogeny was constructed using maximum-likelihood inference with RAxML-NG 0.9.0 (85) and the GTR + G + I evolutionary model. For the iOTUs tree, bootstraps converged after 1,150 replications with 3% cutoff, while for the tree with putative novel isolates it did after 1,450 replications with 2% cutoff. The tree with putative novel isolates was plotted with iTOL v.6 (86).

Compositional and statistical analyses

All analyses were carried out with the R software v.R 4.1.3 (87) and RStudio software v.1.3.1093 (88). Data manipulation was carried out mostly using packages tidyverse v.1.3.1 (89) and qdap v.2.4.3 (90), and plots were created in ggplot2 v.3.3.5 (91). An iOTU table was generated with 99%-clustered isolate 16S rRNA gene sequences. Normalized and rarefied tables were used in some of the subsequent analyses. To generate these rarefied tables, an iOTU table was first grouped by treatment and then rarefied to the

lowest sampling effort (80 isolates in the DL treatment at t_0) with 1,000 permutations with the package EcolUtils v.0.1 (92).

We determined whether culture media, season, and treatment shaped the composition of isolates at the class level. To compare iOTU composition between culture media, a proportional Venn diagram was plotted with package VennDiagram v.1.7.3 (93). To estimate sampling effort in each culture medium and season, rarefaction curves were performed with package vegan v.2.5.7 (94) using non-normalized iOTU tables. To estimate sampling effort across treatments, rarefaction curves were inferred with both non-normalized and rarefied iOTU tables. A PCoA based on Bray-Curtis distances followed by envfit analysis was carried out to test β -diversity across culture media, seasons, and treatments with package vegan v.2.5.7. α -diversity, richness, and the Chao1 estimator (95) were computed using the non-normalized iOTU table, while Shannon indices (96), FPD (97), and standardized effect size mean nearest taxon distance (98) were calculated with the normalized table. Packages ape v.5.6.2 (99) and picante v.1.8.2 (100) were used in order to infer FPD of the iOTUs tree. To test for differences between culture media, seasons, and treatments in all these indices, ANOVA tests and Tukey's post hoc tests were carried out with package stats v.4.1.3. To test differences in culturability across culture media, Wilcoxon rank-sum test was performed with package stats after rejecting normal distribution with Shapiro-Wilk test from the same package. Welch's t test was used to test significant differences in proportions of cultured reads between t_0 and t_f of experiments (only when both samples had more than one replicate). Fisher's exact test was performed to test whether some genera were more likely to be isolated in certain treatments using the non-normalized iOTU table and package stats v.4.1.3. When multiple testing, P -values were adjusted with the Benjamini-Hochberg FDR method (101). All statistical tests were made with the false-discovery rate set to 0.05.

Comparison of isolates with ASVs

The 100%-clustered full 16S rRNA gene sequences of the isolates (ziOTUs) were compared to the ASVs defined from the V4-V5 region of the 16S rRNA gene using BLASTn v.2.12.0+ (84). Those ASVs that presented 100% similarity with one or more ziOTUs were considered as "cultured." An ASV table was used to infer the rank and abundance of each cultured and uncultured taxa by treatment and season, at times t_0 and t_f . Additionally, the fraction of cultured taxa was calculated also for each treatment and season at times t_0 and t_f . The abundances used for these calculations were the means of all sample replicates. The closest neighbor to our isolates and ASVs was inferred searching our ziOTUs sequences against the NCBI 16S ribosomal RNA database (downloaded on 2022/03/01) using BLASTn. To calculate 16S amplicon sequencing abundances, corrected by their 16S rRNA gene copies, reads of each ASV were divided by their mean 16S rRNA gene copies by class except for SAR11, which was treated as a separate category. 16S rRNA gene copy numbers were obtained from rrnDB v.5.8 (67).

ACKNOWLEDGMENTS

We want to thank Núria Vigués and Verónica Melgarejo for their role in maintaining the laboratory in good shape and managing administrative procedures. We would also like to thank Mireia Burnat, Helena Catena, Adrià Auladell, Marta Sebastián, Carolina Marín-Vindas, Cèlia Marrasé, Montse Sala, Vanessa Balagué, and everyone else who participated in the REMEI experiments. We also thank the Marine Bioinformatics Service (MARBITS) of the Institut de Ciències del Mar—CSIC for having provided the computing power needed to perform some of the bioinformatic analyses.

This research was supported by grant RTI2018-101974-B-C22 funded by the Spanish Ministry of Economy and Competitiveness and grants TM2015-70340-R, RTI2018-101025-B-100 and PID2021-125469NB-C32 funded by the Spanish Ministry of Science and Innovation. This work acknowledges the Severo Ochoa Centre of Excellence accreditation

(CEX2019-000928-S). Xavier Rey-Velasco was supported by Spanish FI-SDUR and FPU grants.

AUTHOR AFFILIATIONS

¹Institut de Ciències del Mar (ICM-CSIC), Barcelona, Catalonia, Spain

²Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA-CSIC), Barcelona, Catalonia, Spain

³Centro Oceanográfico de Málaga, Instituto Español de Oceanografía, (IEO-CSIC), Fuengirola, Málaga, Spain

⁴Departament de Genètica i Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Spain

AUTHOR ORCID*s*

Xavier Rey-Velasco  <http://orcid.org/0000-0002-6970-6234>

Josep M. Gasol  <http://orcid.org/0000-0001-5238-2387>

Olga Sánchez  <http://orcid.org/0000-0003-1254-012X>

FUNDING

Funder	Grant(s)	Author(s)
Ministerio de Economía y Competitividad (MEC)	RTI2018-101974-B-C22	Ona Deulofeu Isabel Sanz-Sáez Clara Cardelús Isabel Ferrera Josep M. Gasol Olga Sánchez Xavier Rey-Velasco
Ministerio de Ciencia e Innovación (MCIN)	TM2015-70340-R, RTI2018-101025-B-100	Xavier Rey-Velasco Ona Deulofeu Isabel Sanz-Sáez Clara Cardelús Isabel Ferrera Josep M. Gasol Olga Sánchez
Ministerio de Educación y Formación Profesional (MEFP)	FPU20/01625	Xavier Rey-Velasco
Government of Catalonia Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR)	2020 FISDU 00051	Xavier Rey-Velasco

AUTHOR CONTRIBUTIONS

Xavier Rey-Velasco, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft | Ona Deulofeu-Capo, Data curation, Writing – review and editing | Isabel Sanz-Sáez, Supervision, Writing – review and editing | Clara Cardelús, Project administration, Writing – review and editing | Isabel Ferrera, Writing – review and editing | Josep M. Gasol, Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review and editing | Olga Sánchez, Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review and editing

DATA AVAILABILITY

All supplemental figures and tables can be found on GitHub (<https://github.com/x-rv/Manuscript-2023>). The 16S rRNA gene sequences of the isolates obtained in this study were deposited in GenBank under accession numbers [OP342842](#) to [OP344484](#). Amplicon sequencing data of the V4-V5 region of the 16S rRNA gene used in this study are publicly available in the European Nucleotide Archive under BioProject [PRJEB60085](#).

REFERENCES

- Giovannoni S, Stingl U. 2007. "The importance of culturing bacterioplankton in the "omics" age". *Nat Rev Microbiol* 5:820–826. <https://doi.org/10.1038/nrmicro1752>
- Parker CT, Tindall BJ, Garrity GM. 2019. International code of nomenclature of prokaryotes. *Int J Syst Evol Microbiol* 69:S1–S111. <https://doi.org/10.1099/ijsem.0.000778>
- Staley JT, Konopka A. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu Rev Microbiol* 39:321–346. <https://doi.org/10.1146/annurev.mi.39.100185.001541>
- Martiny AC. 2019. High proportions of bacteria are culturable across major biomes. *ISME J* 13:2125–2128. <https://doi.org/10.1038/s41396-019-0410-3>
- Steen AD, Crits-Christoph A, Carini P, DeAngelis KM, Fierer N, Lloyd KG, Cameron Thrash J. 2019. High proportions of bacteria and archaea across most biomes remain uncultured. *ISME J* 13:3126–3130. <https://doi.org/10.1038/s41396-019-0484-y>
- Lloyd KG, Steen AD, Ladau J, Yin J, Crosby L, Neufeld JD. 2018. Phylogenetically novel uncultured microbial cells dominate earth microbiomes. *mSystems* 3. <https://doi.org/10.1128/mSystems.00055-18>
- Kaeberlein T, Lewis K, Epstein SS. 2002. "Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment". *Science* 296:1127–1129. <https://doi.org/10.1126/science.1070633>
- Steinert G, Whitfield S, Taylor MW, Thoms C, Schupp PJ. 2014. Application of diffusion growth chambers for the cultivation of marine sponge-associated bacteria. *Mar Biotechnol* 16:594–603. <https://doi.org/10.1007/s10126-014-9575-y>
- Ingham CJ, Sprengels A, Bomer J, Molenaar D, van den Berg A, van Hylckama Vlieg JET, de Vos WM. 2007. The micro-petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. *Proc Natl Acad Sci U S A* 104:18217–18222. <https://doi.org/10.1073/pnas.0701693104>
- Wheatley SK, Cartmell C, Madadian E, Badr S, Haltli BA, Kerr RG, Ahmadi A. 2022. Microfabrication of a micron-scale microbial-domestication pod for in situ cultivation of marine bacteria. *RSC Adv* 12:28123–28127. <https://doi.org/10.1039/d2ra05420e>
- Ma L, Kim J, Hatzepichler R, Karymov MA, Hubert N, Hanan IM, Chang EB, Ismagilov RF. 2014. Gene-targeted microfluidic cultivation validated by isolation of a gut bacterium listed in human microbiome project's most wanted taxa. *Proc Natl Acad Sci U S A* 111:9768–9773. <https://doi.org/10.1073/pnas.1404753111>
- Ben-Dov E, Kramarsky-Winter E, Kushmaro A. 2009. An in situ method for cultivating microorganisms using a double encapsulation technique. *FEMS Microbiol Ecol* 68:363–371. <https://doi.org/10.1111/j.1574-6941.2009.00682.x>
- Pope E, Cartmell C, Haltli B, Ahmadi A, Kerr RG. 2022. Microencapsulation and in situ incubation methodology for the cultivation of marine bacteria. *Front Microbiol* 13:958660. <https://doi.org/10.3389/fmicb.2022.958660>
- Alejandre-Colomo C, Harder J, Fuchs BM, Rosselló-Móra R, Amann R. 2020. High-throughput cultivation of heterotrophic bacteria during a spring phytoplankton bloom in the North Sea. *Syst Appl Microbiol* 43:126066. <https://doi.org/10.1016/j.syapm.2020.126066>
- Henson MW, Lanlos VC, Pitre DM, Weckhorst JL, Lucchesi AM, Cheng C, Temperton B, Thrash JC. 2020. Expanding the diversity of bacterioplankton isolates and modeling isolation efficacy with large-scale dilution-to-extinction cultivation. *Appl Environ Microbiol* 86:1–23. <https://doi.org/10.1128/AEM.00943-20>
- Lewis WH, Tahon G, Geesink P, Sousa DZ, Ettema TJG. 2021. Innovations to culturing the uncultured microbial majority. *Nat Rev Microbiol* 19:225–240. <https://doi.org/10.1038/s41579-020-00458-8>
- Pedros-Alíó C. 2012. The rare bacterial biosphere. *Ann Rev Mar Sci* 4:449–466. <https://doi.org/10.1146/annurev-marine-120710-100948>
- Staley JT, Lehmicke LG, Palmer FE, Peet RW, Wissmar RC. 1982. Impact of mount st. helens eruption on ecosystems. *Appl Environ Microbiol* 43:664–670. <https://doi.org/10.1128/aem.43.3.664-670.1982>
- Freitag TE, Klenke T, Krumbein WE, Gerdes G, Prosser JI. 2003. Effect of anoxia and high sulphide concentrations on heterotrophic microbial communities in reduced surface sediments (Black spots) in sandy intertidal flats of the German Wadden sea. *FEMS Microbiol Ecol* 44:291–301. [https://doi.org/10.1016/S0168-6496\(03\)00076-X](https://doi.org/10.1016/S0168-6496(03)00076-X)
- Bartscht K, Cypionka H, Overmann J. 1999. Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. *FEMS Microbiol Ecol* 28:249–259. <https://doi.org/10.1111/j.1574-6941.1999.tb00580.x>
- Jensen PR, Kauffman CA, Fenical W. 1996. High recovery of culturable bacteria from the surfaces of marine algae. *Mar Biol* 126:1–7. <https://doi.org/10.1007/BF00571371>
- Sanz-Sáez I, Salazar G, Sánchez P, Lara E, Royo-Llonch M, Sà EL, Lucena T, Pujalte MJ, Vaqué D, Duarte CM, Gasol JM, Pedros-Alíó C, Sánchez O, Acinas SG. 2020. Diversity and distribution of marine heterotrophic bacteria from a large culture collection. *BMC Microbiol* 20:207. <https://doi.org/10.1186/s12866-020-01884-7>
- Wang M, Noor S, Huan R, Liu C, Li JY, Shi Q, Zhang YJ, Wu C, He H. 2020. Comparison of the diversity of cultured and total bacterial communities in marine sediment using culture-dependent and sequencing methods. *PeerJ* 8:e10060. <https://doi.org/10.7717/peerj.10060>
- Teeling H, Fuchs BM, Becher D, Klockow C, Gardebrecht A, Bennis CM, Kassabgy M, Huang S, Mann AJ, Waldmann J, Weber M, Klindworth A, Otto A, Lange J, Bernhardt J, Reinsch C, Hecker M, Peplies J, Bockelmann FD, Callies U, Gerdtts G, Wichels A, Wiltshire KH, Glöckner FO, Schweder T, Amann R. 2012. Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* 336:608–611. <https://doi.org/10.1126/science.1218344>
- Bouvier T, del Giorgio PA. 2007. Key role of selective viral-induced mortality in determining marine bacterial community composition. *Environ Microbiol* 9:287–297. <https://doi.org/10.1111/j.1462-2920.2006.01137.x>
- Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, Rice S, DeMaere MZ, Ting L, Ertan H, Johnson J, Ferreira S, Lapidus A, Anderson I, Kyrpides N, Munk AC, Detter C, Han CS, Brown MV, Robb FT, Kjelleberg S, Cavicchioli R. 2009. The genomic basis of trophic strategy in marine bacteria. *Proc Natl Acad Sci U S A* 106:15527–15533. <https://doi.org/10.1073/pnas.0903507106>
- Rehnstam A-S, Bäckman S, Smith DC, Azam F, Hagström Å. 1993. Blooms of sequence-specific culturable bacteria in the sea. *FEMS Microbiol Lett* 102:161–166. <https://doi.org/10.1111/j.1574-6968.1993.tb05806.x>
- Simu K, Holmfeldt K, Zweifel UL, Hagström A. 2005. Culturability and coexistence of colony-forming and single-cell marine bacterioplankton. *Appl Environ Microbiol* 71:4793–4800. <https://doi.org/10.1128/AEM.71.8.4793-4800.2005>
- Teeling H, Fuchs BM, Bennis CM, Krüger K, Chafee M, Kappelmann L, Reintjes G, Waldmann J, Quast C, Glöckner FO, Lucas J, Wichels A, Gerdtts G, Wiltshire KH, Amann R. 2016. Recurring patterns in bacterioplankton dynamics during coastal spring algae blooms. *Elife* 5:e11888. <https://doi.org/10.7554/eLife.11888>

30. Gilbert JA, Steele JA, Caporaso JG, Steinbrück L, Reeder J, Temperton B, Huse S, McHardy AC, Knight R, Joint I, Somerfield P, Fuhrman JA, Field D. 2012. Defining seasonal marine microbial community dynamics. *ISME J* 6:298–308. <https://doi.org/10.1038/ismej.2011.107>
31. Shade A, Jones SE, Caporaso JG, Handelsman J, Knight R, Fierer N, Gilbert JA. 2014. Conditionally rare taxa disproportionately contribute to temporal changes in microbial diversity. *mBio* 5:e01371–14. <https://doi.org/10.1128/mBio.01371-14>
32. Sánchez O, Ferrera I, Mabrito I, Gazulla CR, Sebastián M, Auladell A, Marín-Vindas C, Cardelús C, Sanz-Sáez I, Pernice MC, Marrasé C, Sala MM, Gasol JM. 2020. Seasonal impact of grazing, viral mortality, resource availability and light on the group-specific growth rates of coastal mediterranean bacterioplankton. *Sci Rep* 10:19773. <https://doi.org/10.1038/s41598-020-76590-5>
33. Auladell A, Sánchez P, Sánchez O, Gasol JM, Ferrera I. 2019. Long-term seasonal and interannual variability of marine aerobic anoxygenic phototrophic bacteria. *ISME J* 13:1975–1987. <https://doi.org/10.1038/s41396-019-0401-4>
34. Alonso-Sáez L, Balagué V, Sà EL, Sánchez O, González JM, Pinhassi J, Massana R, Pernthaler J, Pedrós-Alió C, Gasol JM. 2007. Seasonality in bacterial diversity in North-West mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. *FEMS Microbiol Ecol* 60:98–112. <https://doi.org/10.1111/j.1574-6941.2006.00276.x>
35. Fuhrman JA, Cram JA, Needham DM. 2015. Marine microbial community dynamics and their ecological interpretation. *Nat Rev Microbiol* 13:133–146. <https://doi.org/10.1038/nrmicro3417>
36. Pinhassi J, Sala MM, Havskum H, Peters F, Guadayol Ò, Malits A, Marrasé C. 2004. Changes in bacterioplankton composition under different phytoplankton regimens. *Appl Environ Microbiol* 70:6753–6766. <https://doi.org/10.1128/AEM.70.11.6753-6766.2004>
37. Teira E, Lekunberri I, Gasol JM, Nieto-Cid M, Alvarez-Salgado XA, Figueiras FG. 2007. Dynamics of the hydrocarbon-degrading cycloclasticus bacteria during mesocosm-simulated oil spills. *Environ Microbiol* 9:2551–2562. <https://doi.org/10.1111/j.1462-2920.2007.01373.x>
38. Teira E, Logares R, Gutiérrez-Barral A, Ferrera I, Varela MM, Morán XAG, Gasol JM. 2019. Impact of grazing, resource availability and light on prokaryotic growth and diversity in the oligotrophic surface global ocean. *Environ Microbiol* 21:1482–1496. <https://doi.org/10.1111/1462-2920.14581>
39. Munoz R, Rosselló-Móra R, Amann R. 2016. Revised phylogeny of bacteroidetes and proposal of sixteen new taxa and two new combinations including rhodothermaeota. *phyl. nov. Syst Appl Microbiol* 39:281–296. <https://doi.org/10.1016/j.syapm.2016.04.004>
40. Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer KH, Whitman WB, Euzéby J, Amann R, Rosselló-Móra R. 2014. Unifying the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Microbiol* 12:635–645. <https://doi.org/10.1038/nrmicro3330>
41. Lekunberri I, Gasol JM, Acinas SG, Gómez-Consarnau L, Crespo BG, Casamayor EO, Massana R, Pedrós-Alió C, Pinhassi J. 2014. The Phylogenetic and ecological context of cultured and whole genome-sequenced planktonic bacteria from the coastal NW mediterranean sea. *Syst Appl Microbiol* 37:216–228. <https://doi.org/10.1016/j.syapm.2013.11.005>
42. Imazaki I, Kobori Y. 2010. Improving the culturability of freshwater bacteria using FW70, a low-nutrient solid medium amended with sodium pyruvate. *Can J Microbiol* 56:333–341. <https://doi.org/10.1139/w10-019>
43. Rodríguez-Valera F, Ventosa A, Juez G, Imhoff JF. 1985. Variation of environmental features and microbial populations with salt concentrations in a multi-pond Saltern. *Microb Ecol* 11:107–115. <https://doi.org/10.1007/BF02010483>
44. Auladell A, Barberán A, Logares R, Garcés E, Gasol JM, Ferrera I. 2022. Seasonal niche differentiation among closely related marine bacteria. *ISME J* 16:178–189. <https://doi.org/10.1038/s41396-021-01053-2>
45. Schauer M, Balagué V, Pedrós-Alió C, Massana R. 2003. Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. *Aquat Microb Ecol* 31:163–174. <https://doi.org/10.3354/ame031163>
46. Mestre M, Höfer J, Sala MM, Gasol JM. 2020. Seasonal variation of bacterial diversity along the marine particulate matter continuum. *Front Microbiol* 11:1590. <https://doi.org/10.3389/fmicb.2020.01590>
47. Allers E, Gómez-Consarnau L, Pinhassi J, Gasol JM, Šimek K, Pernthaler J. 2007. Response of alteromonadaceae and rhodobacteriaceae to glucose and phosphorus manipulation in marine mesocosms. *Environ Microbiol* 9:2417–2429. <https://doi.org/10.1111/j.1462-2920.2007.01360.x>
48. Ionescu D, Bizic-Ionescu M, Khalili A, Malekmohammadi R, Morad MR, de Beer D, Grossart H-P. 2015. A new tool for long-term studies of POM-bacteria interactions: overcoming the century-old bottle effect. *Sci Rep* 5:1–12. <https://doi.org/10.1038/srep14706>
49. Crespo BG, Wallhead PJ, Logares R, Pedrós-Alió C, Martínez-Abarca F. 2016. Probing the rare biosphere of the North-West mediterranean sea: an experiment with high sequencing effort. *PLoS One* 11:e0159195. <https://doi.org/10.1371/journal.pone.0159195>
50. Stefani FOP, Bell TH, Marchand C, de la Providencia IE, El Yassimi A, St-Arnaud M, Hijri M, Hu S. 2015. Culture-dependent and -independent methods capture different microbial community fractions in hydrocarbon-contaminated soils. *PLoS One* 10:e0128272. <https://doi.org/10.1371/journal.pone.0128272>
51. Sogin ML, Morrison HG, Huber JA, Mark Welch D, Huse SM, Neal PR, Arrieta JM, Herndl GJ. 2006. Microbial diversity in the deep sea and the underexplored “rare Biosphere. *Proc Natl Acad Sci U S A* 103:12115–12120. <https://doi.org/10.1073/pnas.0605127103>
52. Sanz-Sáez I, Sánchez P, Salazar G, Sunagawa S, de Vargas C, Bowler C, Sullivan MB, Wincker P, Karsenti E, Pedrós-Alió C, Agustí S, Gojbori T, Duarte CM, Gasol JM, Sánchez O, Acinas SG. 2023. Top abundant deep ocean heterotrophic bacteria can be retrieved by cultivation. *ISME Commun* 3:92. <https://doi.org/10.1038/s43705-023-00290-0>
53. Bartelme RP, Custer JM, Dupont CL, Espinoza JL, Torralba M, Khalili B, Carini P. 2020. Influence of substrate concentration on the culturability of heterotrophic soil microbes isolated by high-throughput dilution-to-extinction cultivation. *mSphere* 5:e00024-20. <https://doi.org/10.1128/mSphere.00024-20>
54. Yang SJ, Kang I, Cho JC. 2016. Expansion of cultured bacterial diversity by large-scale dilution-to-extinction culturing from a single seawater sample. *Microb Ecol* 71:29–43. <https://doi.org/10.1007/s00248-015-0695-3>
55. Connon SA, Giovannoni SJ. 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl Environ Microbiol* 68:3878–3885. <https://doi.org/10.1128/AEM.68.8.3878-3885.2002>
56. Kim S, Park MS, Song J, Kang I, Cho JC. 2020. High-throughput cultivation based on dilution-to-extinction with catalase supplementation and a case study of cultivating acl bacteria from Lake Soyung. *J Microbiol* 58:893–905. <https://doi.org/10.1007/s12275-020-0452-2>
57. Kurm V, van der Putten WH, Hol WHG. 2019. Cultivation-success of rare soil bacteria is not influenced by incubation time and growth medium. *PLoS One* 14:e0210073. <https://doi.org/10.1371/journal.pone.0210073>
58. Jung D, Liu B, He X, Owen JS, Liu L, Yuan Y, Zhang W, He S. 2021. Accessing previously uncultured marine microbial resources by a combination of alternative cultivation methods. *Microb Biotechnol* 14:1148–1158. <https://doi.org/10.1111/1751-7915.13782>
59. Baltar F, Palovaara J, Unrein F, Catala P, Horňák K, Šimek K, Vaqué D, Massana R, Gasol JM, Pinhassi J. 2016. Marine bacterial community structure resilience to changes in protist predation under phytoplankton bloom conditions. *ISME J* 10:568–581. <https://doi.org/10.1038/ismej.2015.135>
60. Riemann L, Steward GF, Azam F. 2000. Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* 66:578–587. <https://doi.org/10.1128/AEM.66.2.578-587.2000>
61. Fandino LB, Riemann L, Steward GF, Long RA, Azam F. 2001. Variations in bacterial community structure during a dinoflagellate bloom analyzed by DGGE and 16S rDNA sequencing. *Aquat Microb Ecol* 23:119–130. <https://doi.org/10.3354/ame023119>
62. Shade A, Gilbert JA. 2015. Temporal patterns of rarity provide a more complete view of microbial diversity. *Trends Microbiol* 23:335–340. <https://doi.org/10.1016/j.tim.2015.01.007>

63. Yooseph S, Neelson KH, Rusch DB, McCrow JP, Dupont CL, Kim M, Johnson J, Montgomery R, Ferriera S, Beeson K, Williamson SJ, Tovchigrechko A, Allen AE, Zeigler LA, Sutton G, Eisenstadt E, Rogers YH, Friedman R, Frazier M, Venter JC. 2010. Genomic and functional adaptation in surface ocean planktonic prokaryotes. *Nature* 468:60–66. <https://doi.org/10.1038/nature09530>
64. Becker JW, Hogle SL, Rosendo K, Chisholm SW. 2019. Co-culture and biogeography of prochlorococcus and SAR11. *ISME J* 13:1506–1519. <https://doi.org/10.1038/s41396-019-0365-4>
65. Hameed A, Shahina M, Lin SY, Lai WA, Hsu YH, Liu YC, Young CC. 2014. *Aquibacter zeaxanthinifaciens* gen. nov., sp. nov., zeaxanthin-producing bacterium of the family *Flavobacteriaceae* isolated from surface seawater, and emended descriptions of the genera *Aestuariibaculum* and *Gaetbulibacter*. *Int J Syst Evol Microbiol* 64:138–145. <https://doi.org/10.1099/ijms.0.052621-0>
66. Winand R, Bogaerts B, Hoffman S, Lefevre L, Delvoeye M, Braekel JV, Fu Q, Roosens NH, Keersmaecker SCD, Vanneste K. 2019. Targeting the 16S rRNA gene for bacterial identification in complex mixed samples: comparative evaluation of second (Illumina) and third (Oxford Nanopore Technologies) generation sequencing technologies. *Int J Mol Sci* 21:298. <https://doi.org/10.3390/ijms21010298>
67. Stoddard SF, Smith BJ, Hein R, Roller BRK, Schmidt TM. 2015. rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res* 43:D593–8. <https://doi.org/10.1093/nar/gku1201>
68. Gómez-Consarnau L, Needham DM, Weber PK, Fuhrman JA, Mayali X. 2019. Influence of light on particulate organic matter utilization by attached and free-living marine bacteria. *Front Microbiol* 10:1204. <https://doi.org/10.3389/fmicb.2019.01204>
69. Ma Y, Ding W, Wang Y, Chen P, Zhou H, Li X, Huang Y, Nan P. 2022. Environmental factors shape the culturable bacterial diversity in deep-sea water and surface sediments from the Mariana Trench. *Microbiology*. <https://doi.org/10.1101/2022.01.13.476188>
70. Massana R, Murray AE, Preston CM, DeLong EF. 1997. Vertical distribution and phylogenetic characterization of marine planktonic archaea in the Santa Barbara channel. *Microbiology* 63:50–56. <https://doi.org/10.1128/aem.63.1.50-56.1997>
71. Parada AE, Needham DM, Fuhrman JA. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 18:1403–1414. <https://doi.org/10.1111/1462-2920.13023>
72. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet j* 17:10. <https://doi.org/10.14806/ej.17.1.200>
73. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 13:581–583. <https://doi.org/10.1038/nmeth.3869>
74. Wright ES. 2016. Using DECIPHER v2.0 to analyze big biological sequence data in R. *The R Journal* 8:352. <https://doi.org/10.32614/RJ-2016-025>
75. Lee YM, Hwang CY, Lee I, Jung YJ, Cho Y, Baek K, Hong SG, Kim JH, Chun J, Lee HK. 2014. *Lacinutrix jangbogonensis* sp. nov., a psychrophilic bacterium isolated from antarctic marine sediment and emended description of the genus *Lacinutrix*. *Int J Gen Mol Microbiol* 106:527–533. <https://doi.org/10.1007/s10482-014-0221-5>
76. Page KA, Connon SA, Giovannoni SJ. 2005. Representative freshwater bacterioplankton isolated from crater lake, Oregon. *Appl Environ Microbiol* 70:22.
77. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>
78. Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>
79. Edgar RC. 2018. Updating the 97% identity threshold for 16S ribosomal RNA Otus. *Bioinformatics* 34:2371–2375. <https://doi.org/10.1093/bioinformatics/bty113>
80. Pruesse E, Peplies J, Glöckner FO. 2012. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28:1823–1829. <https://doi.org/10.1093/bioinformatics/bts252>
81. Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glöckner FO. 2014. “The SILVA and “all-species living tree project (LTP)” taxonomic frameworks”. *Nucleic Acids Res* 42:D643–D648. <https://doi.org/10.1093/nar/gkt1209>
82. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM. 2014. Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* 42:D633–42. <https://doi.org/10.1093/nar/gkt1244>
83. Parks DH, Chuvochina M, Rinke C, Mussig AJ, Chaumeil PA, Hugenholtz P. 2022. GTDB: an ongoing census of bacterial and archaeal diversity through a phylogenetically consistent, rank normalized and complete genome-based taxonomy. *Nucleic Acids Res* 50:D785–D794. <https://doi.org/10.1093/nar/gkab776>
84. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215:403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)
85. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A, Wren J. 2019. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* 35:4453–4455. <https://doi.org/10.1093/bioinformatics/btz305>
86. Letunic I, Bork P. 2021. Interactive tree of life (iTOL) V5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res* 49:W293–W296. <https://doi.org/10.1093/nar/gkab301>
87. R Core Team. 2022. R: a language and environment for statistical computing. Vienna, Austria
88. RStudio Team. 2020. RStudio: Integrated development environment for R. Boston, MA
89. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen T, Miller E, Bache S, Müller K, Ooms J, Robinson D, Seidel D, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H. 2019. Welcome to the tidyverse. *JOSS* 4:1686. <https://doi.org/10.21105/joss.01686>
90. Rinker TW. 2020. qdap: quantitative discourse analysis package. Buffalo, New York.
91. Wickham H. 2016. ggplot2: elegant graphics for data analysis. Springer-Verlag New YorkMedia. <http://link.springer.com/10.1007/978-0-387-98141-3>.
92. Salazar G. 2022. EcolUtils: utilities for community ecology analysis. R package version 0.1.
93. Chen H. 2022. VennDiagram: generate high-resolution venn and euler plots. R package version 1.7.3.
94. Oksanen J, F.Guillaume B, Friendly M, Kindt R, Legendre P, McGlenn D, Minchin PR, O’Hara RB, Simpson GL, Solymos P, Henry M, Stevens H, Szoecs E, Wagner H. 2020. Vegan: community ecology package. R package version 2.5-7
95. Colwell RK, Coddington JA. 1994. Estimating terrestrial biodiversity through extrapolation. *Philos Trans R Soc Lond B Biol Sci* 345:101–118. <https://doi.org/10.1098/rstb.1994.0091>
96. Shannon CE. 1948. A mathematical theory of communication. *BSTJ* 27:379–423. <https://doi.org/10.1002/j.1538-7305.1948.tb01338.x>
97. Faith DP. 1992. Conservation evaluation and phylogenetic diversity. *Biol Conserv* 61:1–10. [https://doi.org/10.1016/0006-3207\(92\)91201-3](https://doi.org/10.1016/0006-3207(92)91201-3)
98. Webb CO, Ackerly DD, McPeck MA, Donoghue MJ. 2002. Phylogenies and community ecology. *Annu Rev Ecol Syst* 33:475–505. <https://doi.org/10.1146/annurev.ecolsys.33.010802.150448>
99. Paradis E, Schliep K. 2019. Ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 35:526–528. <https://doi.org/10.1093/bioinformatics/bty633>
100. Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, Blomberg SP, Webb CO. 2010. Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26:1463–1464. <https://doi.org/10.1093/bioinformatics/btq166>
101. Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* 57:289–300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>