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Analysis of PLA-degrading enzyme from metagenome-assembled genomes and single-cell amplified genomes using TreeSAPP

Parsa Abrishamkar, Angelina Ge, Danny Liu, Kathleen Tom, Lori Waugh

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Since the global production of polyester microplastics is expected to grow, there is an urgent need to understand its fate in the ultimate sink: the marine environment. Global temperature rise and temporal oxygen minimum zone spread will likely influence the community composition of marine prokaryotes which adhere to microplastic polyester particles and which carry genes for polyester degradation. Polylactic acid (PLA), a biodegradable polyester derived from renewable resources, is a leading candidate for the replacement of traditional petroleum-based polyesters, with a simple structure to represent degradation capacity for polyesters as a class. To decipher the microbial community capable of polyester degradation in a future ocean scenario, a phylogenetic reference package for PLA-degrading enzymatic activity (PLAase), with sequences for an enzyme known for PLA degradation and promiscuous activity for multiple polyesters, was developed using the Tree-based Sensitive and Accurate Phylogenetic Profiler (TreeSAPP). Metagenome-assembled genomes (MAGs) and single-cell amplified genomes (SAGs) for a seasonally anoxic fjord, Saanich Inlet, were then screened for phyla containing PLAase genes, using the PLAase reference package, at different depths and correlated against oxygen. Overall, our results revealed a widespread phylogenetic distribution of PLAase genes and transcripts. Additionally, total abundance of PLAase-containing phyla was found to vary with depth in the water column, displaying a general decrease in abundance with increasing depth. Certain phyla, most notably Proteobacteria, were higher in relative abundance, and this pattern was consistent throughout the length of the water column, suggesting that these phyla may have more active PLA-degrading roles. Analysis of geochemical data from the Saanich Inlet showed fluctuations in O_2, CH_4, H_2S, NH_4, NO_2, and NO_3 levels with respect to depth. Although no clear trends were noted between these variables and abundance of PLAase-containing bacterial phyla, specific phyla were identified within a seasonally anoxic depth profile, indicating a possible link between PLAase activity and oxic environments.

INTRODUCTION

Earth's climate is constantly changing, and this prompts the transformation of oceanic conditions. For instance, dissolved oxygen concentrations are decreasing in coastal and open ocean waters, leading to the expansion of oxygen minimum zones (OMZs) (1). Biogeochemical cycles of carbon, nitrogen, and sulfur are mediated by microbes in these zones, and contribute to the production of climate active trace gas and fixed nitrogen loss (2, 3). Due to alterations in biological diversity and food web structure, ocean deoxygenation, a phenotype that only continues to grow as a result of climate change, can be detrimental to ecosystem services and functions (1, 2). As such, current models of geochemical cycles in the ocean need to account for these changes. Saanich Inlet, a seasonally anoxic fjord located on the east coast of Vancouver Island in British Columbia, Canada, is a model ecosystem that can be used to study microbial community responses to ocean deoxygenation (1). This is because the fjord is characterized by a recurring seasonal cycle of water column anoxia, after which deep water renewal occurs, allowing for a spatiotemporal profile spanning across various redox states to be established (1). Furthermore, this inlet is composed of a microbial community similar to that of other oceanic OMZs (1).

Microplastics (1 μm to 5 mm) are globally ubiquitous, persistent, and impinging pollutants (4). Since the early 1900's, mass plastic production has accumulated fragmented

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Address correspondence to: https://jemi.microbiology.ubc.ca/

litter, textile laundry-shed fibers, and consumer product-derived microbeads into the marine environment (5, 6). Upwards of thousands of particles per cubic meter, these microplastics (MPs) can be ingested by native zooplankton species and carried into marine food webs (7, 8). In addition to the direct physical effects of MPs on the marine biota, such as suffocation and starvation, MPs can act as vectors of plastic derived chemicals (e.g., plasticizers and dyes) and environmental pollutants (i.e., PBDEs and heavy metals) into the food web (9-11). Indeed, plasticizers, flame retardants, and UV stabilizers, which leach from plastics, have been shown to disrupt the endocrine system in laboratory experiments on humans and salmon (12). Microplastic fibers, dominantly polyethylene terephthalate (PET) polyester, make up the majority of microplastic particles found in the marine environment of the coastal NW Pacific (7, 13). PET polyester is a petroleum-based polymer with a long degradation rate in the environment, on the timescale of hundreds of years (14), and is the most used synthetic material in the textile industry. Extensive research has been dedicated to understanding the weathering mechanism of PET during UV radiation, temperature, and microbial exposure to predict the fate of this polymer as it further accumulates into the marine environment (14-17). From this work, researchers have found enzymes in the environment which are capable of cleaving the ester bond in the polymer, accelerating their degradation (16-18). These enzymes are theorized to have evolved due to the presence of naturally made (by bacteria in the marine environment) polyester plastics (PHB) (19), but have promiscuity for other polyester types, such as modern human-derived PET plastics (20). These particles are also a hotspot for horizontal gene transfer, given their biofilm, which is known to sustain high bacterial densities and metabolic activity (21). This leads to speculation about the prevalence of polyester-degrading enzymes within marine microbial communities and their phylogenetic assignments, considering the fitness advantage of sorbing onto a carbon-based particle as a potential carbon source.

Another polyester polymer, polylactic acid (PLA), has received notable attention as a leading candidate for replacing petroleum-based polyester plastics in the textile industry, given its biodegradable property and being sourced from renewable sources. In fact, global bioplastics production is increasing almost 40% annually with PLA and its copolymers occupying over 20% of the bioplastics market (18). Taking into account its projected use in consumer products into the future, understanding the fate of this leading biodegradable polymer in the marine environment, all pollutions' final basin, is immensely important.

Given its simple structure as a foundational polyester with only one methyl branch, PLA is an ideal candidate to represent the degradation capacity of the marine microbial community for polyesters as a polymer class, which includes PET. In fact, some of the enzymes that have displayed the capacity to degrade PLA (PLAase enzymes) have also already been studied with PET and displayed the capacity to degrade PET (PETase enzymes): the carboxylesterase EC 3.1.1.1 and cutinase enzyme EC 3.1.1.74 (20). Carboxylesterase EC 3.1.1.1 degrades polyesters by the following reaction:

$$\text{a carboxylic ester} + H_2O \leftrightarrow \text{an alcohol} + \text{a carboxylate} \quad (1)$$

This study aims to gain novel insight into the promiscuous polyester-degrading enzyme 3.1.1.1's capacity to degrade a representative polyester polymer within the Saanich Inlet as a model marine ecosystem with varying oxygenated conditions, enabling us to better understand how future polyester plastic consumer products will degrade in the future deoxygenated deep ocean.

METHODS AND MATERIALS

Saanich Inlet Metagenome-Assembled Genomes (MAGs) data collection and processing. The metagenomic data were acquired as part of a compendium of multi-omic sequence data acquired from the Saanich Inlet water column (2). Water samples containing the genomic data were collected at depths 10, 100, 120, 135, 150, 165 and 200 m in August 2012 at sample collection station S3, Cruise 72. DNA and RNA samples were extracted from the samples and the RNA samples were reversed transcribed into cDNA. The DNA and cDNA samples were used to generate shotgun Illumina libraries. Further sampling and sequencing details are described by Hawley *et al*. in the original publication of the

compendium (2). After quality control and filtering, the reads were assembled into contigs and MAGs were subsequently generated from the metagenome assemblies. The MAGs were then run through GTDB-TK v1.4.0 classify workflow with the reference data version r95 (22). The resulting 219 bins were updated with their Sample IDs in their header and they were concatenated into a single file.

Saanich Inlet Single-Cell Amplified Genomes (SAGs) data collection and processing. Water samples were collected from Saanich Inlet in August 2011. The samples selected for sorting and sequencing were from 100m, 150m, and 185m depth. Microbial cells were sorted from these samples and nucleic acid sequences from each cell were extracted and amplified. The generated SAGs were screened for their small subunit ribosomal rRNA gene (SSU rRNA) via real-time PCR and the resulting amplified sequences were queried against the SILVA database v138.1 (23) with blastn, from BLAST+ v2.9.0 (24). The amplified products were then sequenced and had their adaptors removed and the reads were then assembled into SAGs. SAGs with greater than 5% contamination were decontaminated. The remaining SAGs that were estimated to have >50% completeness and <10% contamination were selected for downstream analyses. These SAGS were then run through GTDB-TK v1.4.0 classify workflow (22) and they were concatenated into a single file.

Acquiring reference sequence data from PAZy. Reference sequence data with EC number 3.1.1.1 were acquired from the Plastics-Active Enzymes Database (PAZy) of esterase (20). The data included full genomic sequences of esterase from select bacteria (18, 25-27) as well as marine metagenomic data sourced from the Mediterranean and Barents Sea (28).

Gene-centric analysis using TreeSAPP. Functional and taxonomic annotation of the sequences was conducted using the Tree-based Sensitive and Accurate Phylogenetic Profiler (TreeSAPP) software version 0.11.3 (29). The initial step of the TreeSAPP workflow involved the treesapp create command to create a seed reference package for PLA enzymes using the sequences compiled from PAZy. The purity of this reference package was then tested with treesapp purity, in which the TIGRFAM seed sequences from the TIGRFAM database (30) were used to ensure the reference package did not contain any mis-annotated sequences. The reference package tree inferred by treesapp create was written to a file in Newick format using the subcommand treesapp package, and the resulting phylogenetic tree was visualized in Interactive Tree Of Life version 6 (iTOL v6) (31). Following this, the seed reference package was updated with the MAGs and SAGs collected from Saanich Inlet. The MAGs were first classified using treesapp assign and then added to the seed reference package using treesapp update. Using the MAGs-updated reference package, the two aforementioned commands were repeated for the SAGs, and the updated reference package was visualized in iTOL v6. Lastly, the treesapp abundance subcommand was carried out to analyze the relative abundance of reads by calculating transcripts per million (TPM). See Supplementary Figure 1 for the phylogenetic tree from the seed reference package and Supplementary File 2 for full commands.

Analysis of geochemical data in R. Analysis of geochemical, metagenomic and metatranscriptomic data was conducted in R version 4.1.2 and utilized the ggplot2, dplyr and tidyr packages. See Supplementary File 3 for full commands.

RESULTS

PLAase-encoding bacteria are phylogenetically diverse and vary in abundance with depth. To assess the phylogeny of PLAase gene-containing phyla, we generated phylogenetic trees in iTOL (31) (**Figure 1**). This analysis showed that certain phyla contained more species with PLAase genes than other phyla. For instance, a high amount of *Proteobacteria* sp. were observed but only one species in the phylum Firmicutes (*Paenibacillus amylolyticus*) was found in both the metagenomics (**Figure 1a**) and metatranscriptomics (**Figure 1b**) phylogenetic trees. Various phylogenetic placements (orange bubbles) were also observed between the phyla in Saanich Inlet.

Furthermore, phyla abundance was calculated at various depths of the Saanich Inlet (**Figure 1**). Here, we see that phyla abundance varies with depth and also across species in a phylum. While some *Proteobacteria* sp. had no hits at any of the Saanich Inlet depths, other *Proteobacteria* sp. displayed hits at all of the depths. Interestingly, *P. amylolyticus* had the highest abundance in all of the depths.

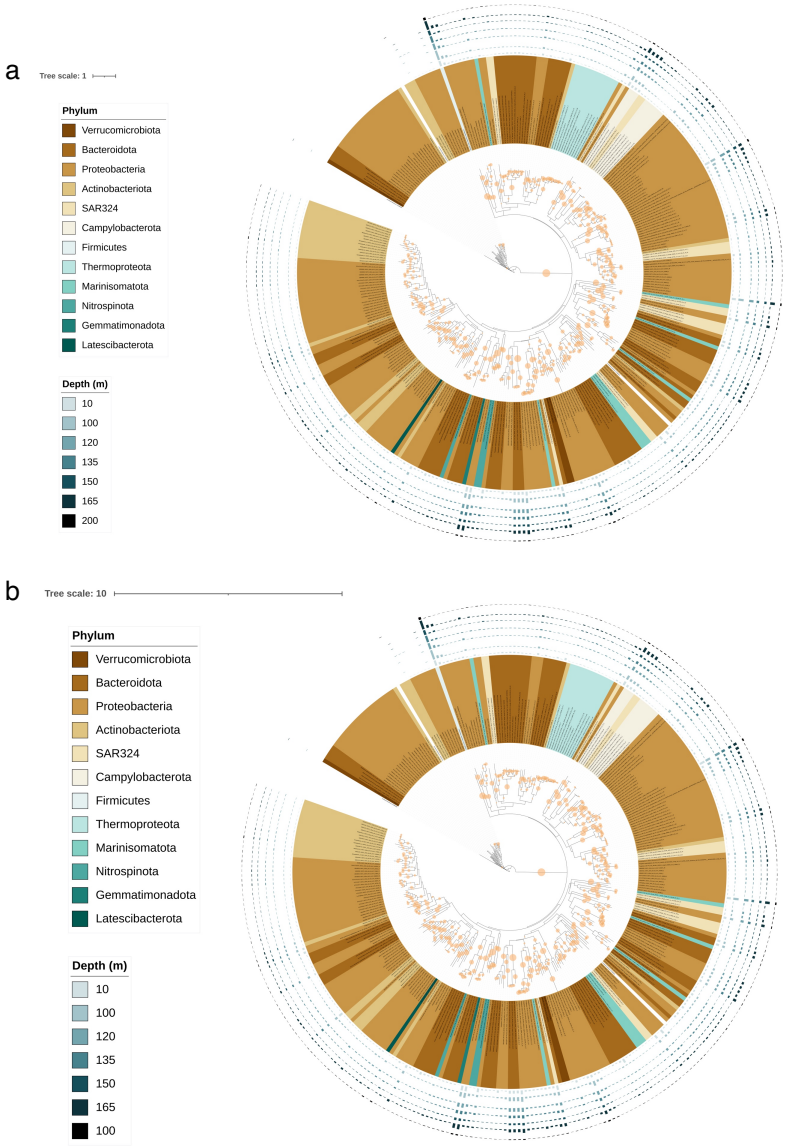

FIG. 1 Phylogeny of PLAase gene-containing phyla after functional annotation. Phylogenetic trees of phyla containing PLAase genes were rooted at the midpoint using iTOL for a) metagenomics and b) metatranscriptomics. Abundance of phyla at various depths of the Saanich Inlet are shown. Orange bubbles display the midpoint of branches where 10399 query proteins from UniProt were placed. Abundance of phyla containing PLAase genes varies with depth and across species within a phylum. The trees can be viewed more interactively and with greater resolution at https://itol.embl.de/shared/MICB425_g3.

PLA degradation potential is highest at the surface level but phyla abundance varies with depth. Since we observed that phyla abundance varies with Saanich Inlet depth, we wanted to quantitatively confirm these findings using relative abundance plots for metagenomics (**Figure 2a**) and metatranscriptomics across various depths (**Figure 2c**). As such, we observed that phyla abundance indeed varied with depth, but the relative abundance was generally consistent. Across all depths, the phylum Proteobacteria displayed the highest relative abundance in comparison to the other phyla, while uncultured phyla displayed the second highest relative abundance.

Additionally, total abundance plots for metagenomics (**Figure 2b**) and metatranscriptomics across various depths (**Figure 2d**) were generated to investigate the total amount of phyla containing PLAase genes at each depth. Both plots showed that phyla abundance, and thus PLA degradation potential, was highest at surface-level depths and generally decreased with increasing depth. However, interestingly, this trend was not seen at the 165-meter depth, which displayed higher phyla abundance than the 150-meter depth.

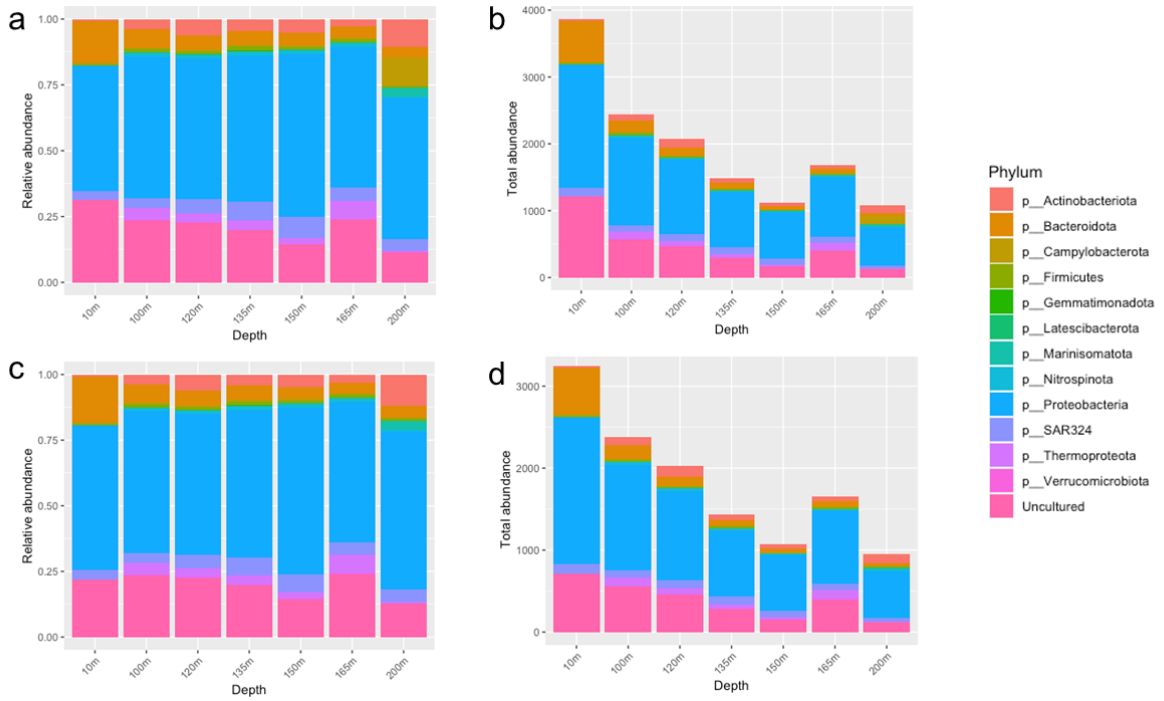

FIG. 2 PLA degradation potential is highest at the surface level but phyla abundance varies with depth. Relative abundance of phyla containing PLAase genes was calculated at various depths of the Saanich Inlet for a) metagenomics and c) metatranscriptomics. Certain phyla are consistently more abundant than others across all depths. Total abundance in transcripts per million (TPM) was calculated for the phyla at various depths for b) metagenomics and d) metatranscriptomics. Total abundance varies according to depth.

O_2 concentrations are correlated with Proteobacteria abundance. Using the relative and total abundance data, we were then interested whether there was any correlation between microbial abundance and the concentrations of specific chemical molecules at various depths (**Figure 3**). This is important with respect to microbial abundance and PLAase activity; the large range of concentration for each of the chemicals may result in conditions where PLAase carries out depolymerization more efficiently or facilitates the survival of PLAase-expressing bacteria. More specifically, when comparing the concentration of O_2 with microbial abundance, the decreasing O_2 concentration with increasing depths is also correlated with an increase in proteobacterial abundance in both the metagenomic and metatranscriptomic readings (**Figure 4**). However, as Proteobacteria encompass a large variety of classes, further analysis can be conducted whether the dominant classes are aerobic or anaerobic.

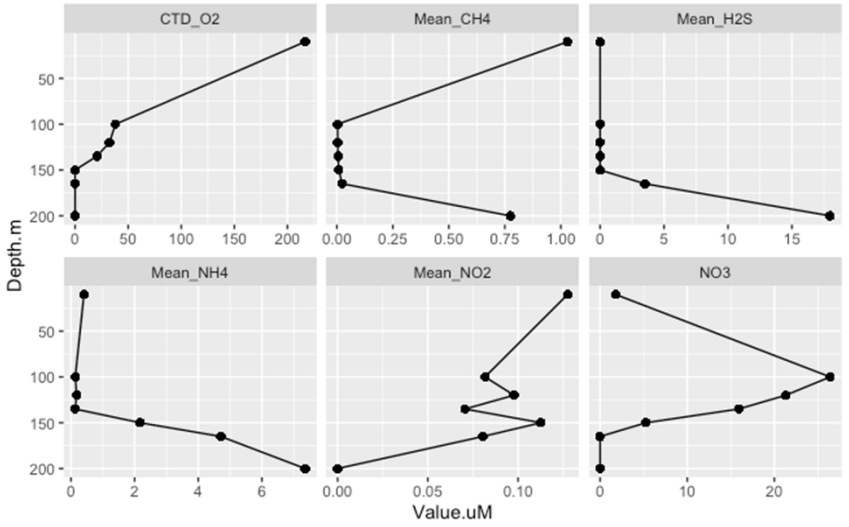

FIG. 3 The Saanich Inlet dataset shows varying chemical measurements at different depths. Both NH_4 and H_2S experience an increase in concentration as depth increases, while NO_2 and O_2 experience a decrease. Both CH_4 and NO_3 experience a large spike in their concentrations at ~100m; the spike in CH_4 is a sharp decrease while the spike in NO_3 is a large increase in concentration.

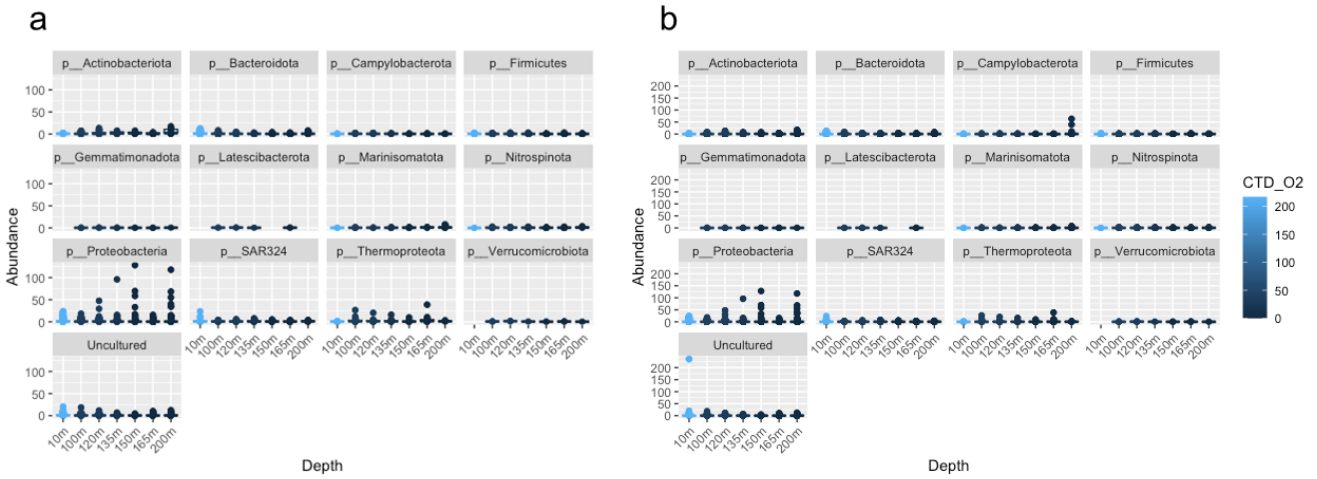

FIG. 4 O_2 concentrations are slightly correlated with Proteobacteria abundance. At lower depths, O_2 concentrations are higher while Proteobacteria abundance and PLAase expression is higher as seen from the low abundance in the a) MetaG and b) MetaT datasets respectively.

DISCUSSION

***Paenibacillus amylolyticus* strain TB-13 has the highest abundance of PLAase genes at all depths.** Based on the phylogeny and PLAase abundance values depicted by the metagenomic and metatranscriptomic trees (**Figure 1**) it appears that the species *P. amylolyticus* strain TB-13 has the highest abundance values for PLAase genomic and transcriptomic abundances at all depths, when compared to other species in the trees. This strain has been reported to be able to degrade various plastics (including PLA) and it is speculated that it performs this degradation via expression of proteases and esterases (32). Another study by Akutsu-Shigeno *et al.* in 2003 reported that cloning the PLA depolymerase gene from *P. amylolyticus* strain TB-13 into *E. coli* resulted in production of purified recombinant PLA depolymerase that showed degradation activity towards biodegradable polyesters such as PLA (26). Hence, the strong presence of *P. amylolyticus* strain TB-13 at all depths in our metagenomic and metatranscriptomic tree is in alignment with the reported PLA-degrading capacity of this strain. The assignment of a strain with known polyester degradation capacity within the Saanich Inlet updated PLAase reference package indicates that the PLAase reference package is successfully identifying PLA-degrading organisms.

Expression of PLAase genes is highest at surface-level depths. Our results obtained from investigating the total abundance of PLAase showed a general pattern of decreased abundance of PLAase-containing phyla with increasing depth (**Figure 2b, Figure 2d**). This trend was noted for both metagenomic and metatranscriptomic data, indicating that surface-level depths are where PLAase-containing microbial communities are more prevalent and where their expression of PLAase genes is greatest. This latter observation further suggests that surface-level depths are also where PLA-degrading microorganisms most exert their PLA-degrading functions. The reason behind these findings is largely unknown, but given that the presence and abundance of microorganisms are closely linked to environmental conditions in marine environments, certain abiotic factors in the Saanich Inlet may be playing a role. For instance, oxygen content, which varies with depth in the Saanich Inlet (**Figure 3**), is known to be closely tied to the abundance and presence of certain microbes in marine environments (33). In the Saanich Inlet, oxygen levels decrease as depth increases (**Figure 3**); abundance of PLAase-containing bacteria also decreases as depth increases (**Figure 2**). These concurrent findings may suggest that oxygen levels may be linked to the abundance of PLA-degrading bacteria. Amongst the apparent heightened presence of biodegradation capabilities by microbes in surface waters, other plastic degradation pathways, including UV radiation (34), hydrolysis (35), and physical weathering (36), are also greatest in surface waters, where irradiance, temperatures, and turbulence are greatest within the water column. Given abiotic weathering is known to amplify biodegradation (16), it appears that plastic degradation is most prominent in surface waters. However, the Saanich Inlet dataset does not include

sediment samples. Oxic and anoxic marine sediments are a known hotspot for PLAase enzyme-carrying prokaryotes, acting as the ultimate sink to these particles (37).

Certain phyla may have larger roles in PLA degradation. Our analysis into the relative abundance of PLAase-containing phyla revealed a number of findings. The relative abundance profiles of the metagenomic (**Figure 2a**) and metatranscriptomic (**Figure 2c**) data exhibited general consistency both between each other and across depths. For instance, the phylum Proteobacteria had the highest relative abundance at all depths, suggesting that members of the Proteobacteria group may represent an important source of PLA degraders. Although there is an absence of studies examining PLA degradation in Proteobacteria in a marine environment, Proteobacteria are thought to contribute to PLA biodegradation in soils (38, 39). In addition, in recent research looking more broadly at the capability of microbes to degrade plastics in general, the phylum Proteobacteria frequently shows the highest relative abundance (40). As seen in **Figure 2**, uncultured microbes, meaning microbes which were unassigned, were found to constitute the second highest relative abundance, which may be explained by the fact that three of the seven PLAase-associated sequences used to produce the PLAase package seed tree were not assigned phylogenetic lineages, only labeled as marine metagenomes (28). Thus, assignments to this homogen may have resulted in a phylogenetic tree skewed for uncultured microbes. This indicates that more work is necessary to assign microbes with PLA-degrading abilities, and to complete the PLAase reference package with assigned organisms. This will likely influence the current movement in the microbial community to determine which organisms carry the capacity to degrade microplastic particles, particularly polyesters (38, 40).

Intriguingly, although our results revealed the presence of the phylum Actinobacteria, its relative abundance was notably low (**Figure 2**). This was unexpected, given previous findings characterizing Actinobacteria as one of the most prominent PLA degraders (40, 41). Our conflicting result may be attributed to the fact that isolation of PLA-degrading Actinobacteria is known to be challenging (41); perhaps Actinobacteria capable of PLA degradation are in actuality more abundant than our data suggest, but cannot be cultured and so are part of the aforementioned group of uncultured microbes. It is also possible that the environmental or geochemical conditions of the Saanich Inlet may be impacting the survivability, and thus abundance, of some Actinobacteria.

Conclusions This study aimed to explore the diversity, abundance, and expression of PLAase degradation within the Saanich Inlet, focusing on the promiscuous polyesterase gene (EC 3.1.1.1). In addition to uncovering a diversity of PLAase-encoding bacteria, our results showed a greater abundance of PLA degraders at surface-level depths and identified specific PLAase-associated phyla within a seasonally anoxic depth profile, indicating that PLAase activity is associated with oxic environments. However, the poor sample resolution within surface depths, and the low number of PLAase-associated and lineage-assigned sequences available to build the reference package, hindered analysis and no clear trends were observed between the geochemical variables of the Saanich Inlet and phyla abundance.

Future Directions Given that the results of this study showed a strong and diverse presence of PLA-degrading bacteria, future studies could investigate the abundance of enzymes or bacteria that degrade other polyesters such as PBS (polybutylene succinate), PBSA (polybutylene succinate-co-butylene adipate), and PET (polyethylene terephthalate), as well as biologically derived polyesters such as PHB to decipher the promiscuity in polyester degradation capacity within the marine environment. These studies could also look into whether there is any overlap between the phylogeny of the PLA-degrading organisms and that of other polyester-degrading bacteria. Furthermore, the seed reference package constructed in this study was based on a total of seven bacterial hosts and/or metagenomic samples, encoded as EC 3.1.1.1 in the PAZy database; therefore, future projects could construct a more extensive PLA seed reference package based by acquiring data from a more diverse set of genomic samples from the PAZy database. In addition, given that the abundance analyses conducted in this study were performed at the phylum level, future projects could improve the run the analyses at higher taxonomic ranks (e.g., species level) in order to gain a

more detailed perspective on specific taxa that may be expressing PLA-degrading genes. Future studies could also analyze the abundance level of bacteria that naturally produce polyesters such as PHB (polyhydroxybutyrate) and investigate whether there is a relationship between abundance of polyester-producing genes and polyester-degrading genes. Furthermore, the diversity of phyla with PLA degradation capacity suggests the presence of horizontal gene transfer, likely amplified within the biofilm, which may be studied in marine microcosms by enriching seawater samples with fluorescently tagged PLAase enzyme bacteria in the presence and absence of microplastic polyester particles (42).

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