Concentration-Dependent PAH Degradation and Gene Expression in Marine *Cycloclasticus* spp.

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Statement of authorship

I hereby declare that I have written the submitted thesis on my own. All references and resources used as well as passages that are taken literally or in terms of content from the works of other authors have been marked as such. I have made a detailed distinction between my own work and the contributions of my cooperation partners in the ”Statement of personal contribution” chapter.

Ich erkläre hiermit, dass ich die zur Promotion vorgelegte Arbeit selbständig verfasst habe. Alle verwendeten Quellen und Hilfsmittel sowie Passagen, die wörtlich oder inhaltlich aus den Werken anderer Autoren entnommen sind, wurden als solche gekennzeichnet. Eine detaillierte Abgrenzung meiner eigenen Leistungen von den Beiträgen meiner Kooperationspartner habe ich im „Statement of personal contribution“ vorgenommen.

Anjela Lucia Vogel

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Summary

Input of polycyclic aromatic hydrocarbon (PAH) contamination in marine ecosystems occurs over several orders of magnitude and microbial degradation is a relevant and arguably crucial attenuation process. However, the remediation of low-dosage PAH contaminations through biodegradation remains largely unstudied. In the presented thesis, the knowledge gap surrounding PAH-degradation activity under different contaminant loads was addressed with a combination of numerical modelling and laboratory experiments covering questions from the dynamics of biochemical pathways to the overall response of environmental systems.
In the first published study presented in this thesis, a process-based numerical model was developed. Biomass growth and naphthalene degradation were simulated for *Cycloclasticus* spp. batch cultures receiving repeated low-dosage naphthalene pulses compared to the conventionally used one-time high-dosage (Vogel et al., 2023a). The results showed that pulsing frequency and dosage concentration impacted the degradation efficiency in the simulated experiments considerably. In consequence, dissolution kinetics dictated biodegradation and biomass growth, making the final biomass concentration of PAH-degrading bacteria alone not a sufficient indicator for the quantification of active biodegradation. Furthermore, a one-time input of a high-naphthalene dose was degraded faster than repeated low dosages, implying that repeated low-dosage input could lead to PAH accumulation in exposed pristine environments. Thus, the interactions of coupled low-concentration pollutant degradation and microbial growth processes were elucidated and the results may have important implications for future bioremediation management of diffuse oil contamination in the marine environment.
For risk assessment and bioremediation monitoring, knowledge of *in situ* degradation rates is imperative. The ratio of mRNA to DNA of functional genes, the professed transcript-to-gene ratio, could be a cultivation-independent, measurement to obtain per-cell degradation rates — potentially even in a high-throughput manner. If successful, such a measure would provide a method for quantifying *in situ* PAH-degradation rates. The second and third studies aimed, therefore, to understand expression of functional genes under various PAH loads, which might help to monitor and predict bioremediation efficiency in the future. It was, however, unknown if the transcription of PAH-degradation biomarker genes could serve as an indicator of active PAH degradation. Using the model PAH degrader *Cycloclasticus pugetii* strain PS-1, substrate-independent expression of three key functional marker genes was found during the degradation of naphthalene, phenanthrene, a combination of both, and no-PAH controls at high concentrations (i.e., 200 mg L^{-1}) using qPCR (Vogel et al., 2023b).

In subsequent RNA-sequencing experiments with high and low naphthalene dosages (i.e., 100 and 30 mg L^{-1}) genetic redundancy was detected as 15 genes encoding for enzymes involved in the initial step of PAH degradation were highly transcribed. Remarkably, some of these were transcribed during active naphthalene degradation, while others were expressed in naphthalene-starvation treatments. Additionally, some genes — including the three functional marker genes from Vogel et al., 2023b — were expressed substrate-independently, regardless of available naphthalene (Vogel et al., 2024). Hence, two distinct enzymatic systems for the naphthalene-degradation pathway were proposed: one where the encoding genes were transcribed substrate-independently resulting in ”background” PAH degradation, and a second system where the encoding genes were transcribed in response to the PAH enabling a ”rapid response” following naphthalene exposure. This hypothesized genetic flexibility might allow highly specialized PAH degraders like *Cyclcoalsticus* spp. to adapt to changing PAH dosages (i.e., concentrations) in a need-based manner. Ultimately, the results imply that the transcription of PAH-marker genes does not necessarily correspond with PAH-degradation activity, and (meta)transcription data should always be evaluated with caution.

In summary, this work has greatly expanded our understanding of the dependence between PAH degradation (activity) and gene expression in marine environments. The presented thesis highlights how an interdisciplinary approach which includes numerical modelling and wet-lab-based studies can close knowledge gaps that are difficult to investigate with more classical experiments. The results show that the so-far-overlooked parameters of PAH dosage and frequency can highly influence biodegradation on multiple levels and, thereby, the fate of contaminants within marine environments.

Zusammenfassung

Der Eintrag von Verunreinigungen durch polyzyklische aromatische Kohlenwasserstoffe (PAK) in Ökosysteme der Meere erfolgt über mehrere Größenordnungen und der mikrobielle Abbau ist ein höchst relevanter und entscheidender Verminderungsprozess. Die Sanierung niedrig dosierter PAK Kontaminationen durch biologischen Abbau ist jedoch noch weitgehend unerforscht. In der vorgestellten Dissertation wurde die Wissenslücke bezüglich der PAK Abbauaktivität unter verschiedenen Schadstoffbelastungen mit einer Kombination aus prozessorientierter, numerischer Modellierung und Laborexperimenten untersucht und Fragen von der Dynamik biochemischer Pfade bis zur Gesamtreaktion von Umweltsystemen auf PAKs behandelt.

In der ersten veröffentlichten Studie, die in dieser Arbeit vorgestellt wird, wurde ein prozessbasiertes numerisches Modell entwickelt. Biomassenwachstum und Naphthalenabbau wurden für *Cycloclasticus* spp. simuliert. Batch-Kulturen, die wiederholte Naphthalenimpulse in niedriger Dosierung erhielten, wurden mit der herkömmlich verwendeten einmaligen Hochdosis verglichen (Vogel et al., 2023a). Die Ergebnisse zeigten, dass die Pulsfrequenz und die Dosierungskonzentration die Abbaueffizienz in den simulierten Experimenten erheblich beeinflussten. Folglich bestimmte die Löslichkeitskinetik den biologischen Abbau und das Biomassenwachstum, sodass die endgültige Biomassen- konzentration der PAK abbauenden Bakterien allein kein ausreichender Indikator für die Quantifizierung des aktiven biologischen Abbaus war. Darüber hinaus wurde die einmalige Gabe einer hohen Naphthalendosis schneller abgebaut als die wiederholte Gabe niedriger Naphthalendosen. Dies deutet darauf hin, dass die wiederholte Gabe einer niedrigen Naphthalendosis zu einer Anreicherung von PAKs in unberührten Umgebungen führen könnte. Auf diese Weise wurden die Wechselwirkungen zwischen dem gekoppelten Abbau von Schadstoffen in geringer Konzentration und mikrobiellen Wachstumsprozessen aufgeklärt, und die Ergebnisse könnten wichtige Auswirkungen auf das zukünftige biologische Sanierungsmanagement diffuser Ölverschmutzung in der Meeresumwelt haben.

Für die Risikobewertung und die Überwachung der biologischen Sanierung ist die Kenntnis der *in situ* Abbauraten unerlässlich. Das Verhältnis von mRNA zu DNA funktioneller Gene, das sogenannte Transkript-zu-Gen-Verhältnis, könnte eine kultivierungsunabhängige Messung sein, um Abbauraten pro Zelle zu ermitteln — möglicherweise sogar als Hochdurchsatz Methode. Im Erfolgsfall würde eine solche Messung eine Methode zur Quantifizierung der *in situ* PAK Abbauraten liefern. Ziel der zweiten und dritten Studie war es daher, das Ver-

ständnis der Genexpression von Biomarkergenen unter verschiedenen PAK Belastungen zu verbessern. Dies könnte dazu beitragen, die Wirksamkeit der Bioremediation in Zukunft zu überwachen und vorherzusagen. Jedoch war es bisher nicht bekannt, ob die Transkription von PAK Biomarkergenen als Indikator für den aktiven PAK Abbau dienen könnte. Durch Verwendung von qPCR wurde im Modellstamms des PAK Abbauers *Cycloclasticus pugetii* strain PS-1 eine substratunabhängige Expression von drei wichtigen funktionellen Markergenen während des Abbaus von Naphthalen, Phenanthren, einer Kombination aus beiden, und Nicht-PAK-Kontrollen in hohen Konzentrationen (d. h. 200 mg L^{-1}) festgestellt (Vogel et al., 2023b). In anschließenden RNA Sequenzierungsexperimenten mit hohen und niedrigen Naphthalendosierungen (d. h. 100 und 30 mg L^{-1}) wurde eine genetische Redundanz festgestellt, da 15 Gene, die für Enzyme kodieren, die am ersten Schritt des PAK Abbaus beteiligt sind, hoch transkribiert wurden. Bemerkenswerterweise wurden einige davon während des aktiven Naphthalenabbaus transkribiert, während andere bei Experimenten mit Naphthalenmangel transkibiert wurden. Darüber hinaus wurden einige Gene —– darunter die drei funktionellen Markergene aus Vogel et al., 2023b —– unabhängig vom verfügbaren Naphthalen substratunabhängig exprimiert (Vogel et al., 2024). Daher wurden zwei unterschiedliche enzymatische Systeme für den Naphthalen Abbauweg vorgeschlagen: eines, bei dem die kodierenden Gene substratunabhängig transkribiert wurden, was zu einem „Hintergrund“ PAK Abbau führte, und ein zweites System, bei dem die kodierenden Gene als „Schnelle Reaktion“ auf das PAK transkribiert wurden. Diese hypothetische genetische Flexibilität könnte es hochspezialisierten PAK Abbauern wie *Cyclcoalsticus* spp. ermöglichen, sich bedarfsgerecht an veränderte PAK Dosierungen (also Konzentrationen) anzupassen. Letztendlich deuten die Ergebnisse darauf hin, dass die Transkription von PAK Markergenen nicht unbedingt mit der PAK Abbauaktivität korrespondiert und (Meta-)Transkriptionsdaten immer mit Vorsicht ausgewertet werden sollten.

Zusammenfassend hat diese Arbeit unser Verständnis des konzentrationsabhängigen PAK Abbaus und der Genexpression in Ökosystemen des Meeres erheblich erweitert. Die hier vorgestellte Dissertation zeigt, wie ein interdisziplinärer Ansatz, der numerische Modellierung und nasslaborbasierte Studien umfasst, Wissenslücken schließen kann, die mit klassischeren Experimenten nur schwer zu untersuchen sind. Die Ergebnisse zeigen weiterhin, dass die bisher übersehenen Parameter der Dosierung und Häufigkeit von PAK Eintrag den biologischen Abbau auf mehreren Ebenen und damit das Problem von Schadstoffen in der Meeresumwelt stark beeinflussen können.

Published articles

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A.L. Vogel, K. J. Thompson, S. Kleindienst, C. Zarfl (2023a). Dosage concentration and pulsing frequency affect the degradation efficiency in simulated bacterial PAH degrading cultures. *Environ Sci Pollut Res*, **30**, 59813–59825, https://doi.org/10.1007/s11356-023-26546-9

A. L. Vogel, K. J. Thompson, C. B. App, D. Straub, T. Gutierrez, F. E. Löffler, S. Kleindienst (2023b). Substrate-independent expression of key functional genes in *Cycloclasticus pugetii* strain PS-1 limits their use as PAH biodegradation markers. *Front Microbiol*, **14**, https://doi.org/10.3389/fmicb.2023.1185619

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A. L. Vogel, K. J. Thompson, D. Straub, F. Musat, T. Gutierrez, S. Kleindienst (2024). Genetic redundancy in the naphthalene-degradation pathway of *Cycloclasticus pugetii* strain PS-1 enables response to varying substrate concentrations. *FEMS Microbiol Ecol*, **100**, 6, fiae060, https://doi.org/10.1093/femsec/fiae060

Statement of personal contribution

This PhD Thesis was funded by the Emmy Noether program of the German Research Foundation (DFG, Deutsche Forschungsgemeinschaft - #326028733) granted to Prof. Sara Kleindienst, who was also the project's main advisor and created the initial concept of the presented work. Prof. Christiane Zarfl supported the work as a second advisor and, particularly, Vogel et al., 2023a in close collaboration with her. Unless otherwise stated, experiments were conceptualized and carried out by myself, Dr. Katharine J. Thompson, and Prof. Sara Kleindienst. The discussion and analysis of the obtained results, as well as the drafting of all papers, were accomplished in collaboration with Dr. Thompson and Prof. Kleindienst; for Vogel et al., 2023a also in collaboration with Prof. Zarfl. In detail, the contributions of all co-authors, including myself and other people engaged, are listed below:

Vogel et al., 2023a: This study, in which we modelled the effect of PAH dosage concentration and frequency on biodegradation, was designed by myself and Prof. Christiane Zarfl. Dr. Adrian Mellange was involved in early discussions about model development. The Matlab code for the model was developed by myself and Prof. Zarfl. Results of the simulated scenarios were discussed between myself, Prof. Zarfl, Dr. Katharine J. Thompson, and Prof. Sara Kleindienst. The first version of the manuscript was written by myself and revised by all co-authors.

Vogel et al., 2023b: The experiments for the qPCR-based study on substrate- independent expression of functional marker genes were designed by myself with input from Dr. Adrian Langarica-Fuentes, Dr. Daniel Straub, Dr. Katharine J. Thompson, and Prof. Sara Kleindienst. Primer design and qPCR-method development were conducted by myself with assistance from Franziska Schädler and feedback from Dr. Straub. Lars Grimm helped with the revival of the purchased culture from the ATCC culture collection. Preliminary testing for the pure culture experiments including growth experiments method optimization, and development of a standardized workflow, as well as experimental preparations were carried out by myself while Franziska Schädler kindly assisted with setting up the published pure culture experiments. Experimental sampling and hydrocarbon extraction were performed by myself and GC-MS measurements were performed by Renate Seelig. Two student assistants - Maike Friedel and Kleanthi Kourtaki - helped with DAPI cell counts, DNA and RNA extractions, and qPCR assays, while RNA processing was done by myself. DNA and RNA

samples from Arctic microcosm experiments were provided by Constantin B. App. Data collection, visualization, and preliminary interpretation were done by myself, before results were discussed among Dr. Thompson, Prof. Kleindienst, Prof. Frank E. Löffler, and Prof. Tony Gutierrez. The manuscript was written by myself and revised by all co-authors. Dr. Straub performed statistics as required during the revision of the manuscript and wrote the method descriptions for the statistics section which was revised by all other co-authors.

Vogel et al., 2024: The RNA sequencing experiments, investigating genetic redundancy in the PAH-degradation pathway of *Cycloclasticus* spp. was designed by myself, Dr. Katharine J. Thompson, and Prof. Sara Kleindienst. Experimental planning and preparations were carried out by myself, Franziska Schädler assisted with setting up the experiment. Experimental sampling, DNA and RNA extraction, RNA purification for sequencing, qPCR assays, hydrocarbon extraction, and data visualization were performed by myself. DNA-free RNA was submitted to the Institute for Medical Microbiology and Hygiene (MGM, University of Tübingen) for library preparation and sequencing. Bioinformatic analysis of sequencing data was performed by Dr. Daniel Straub, downstream analysis and visualisation were conducted by myself, and results were interpreted by myself together with Dr. Straub, Dr. Thompson, Prof. Kleindienst, and the remaining co-authors Prof. Florin Musat and Prof. Tony Gutierrez. GC-MS measurements of hydrocarbon extracts were performed by Renate Seelig and the results were interpreted by myself together with Dr. Thompson and Prof. Kleindienst. The manuscript was written by myself and revised by all co-authors. Dr. Straub wrote the method descriptions for bioinformatics which was revised by all other co-authors.

I hereby declare that I have independently written this thesis, that I have only used the indicated references, and that I have not plagiarized any of the text. Vogel et al., 2023a and 2023b have been published in scientific journals. A third manuscript — Vogel et al., 2024 — was under revision prior to submission of the thesis in December 2023, but was published between submission and publication of the thesis.

1. Introduction

1.1 Hydrocarbons in the ocean

1.1.1 Sources of marine hydrocarbon input

Oceans cover approximately 71% of Earth's surface and make up the majority of the hydrosphere (Visbeck, 2018). Therefore, marine ecosystems play an important role in climate regulation, the oxygen and carbon cycles, food supply, and the tourism industry as well as providing a habitat for many species (C. M. Roberts & Hawkins, 1999; Visbeck, 2018). The input of contaminants into the oceans can cause a disturbance to these fragile ecosystems, and the resulting effects can be both dramatic and difficult to predict. Crude oil components are critical contaminants in marine environments due to both their acute and chronic toxicity effects. Therefore, a comprehensive understanding of the processes that impact input, fate, and the effects of crude oil contamination in the ocean is crucial.

Between 0.47 and 8.3 million tons of crude oil compounds, such as hydrocarbons, are estimated to enter the marine environment each year (Fig. 1.1). The sources that discharge the majority of hydrocarbons each year (48%) are natural oil seeps in the seafloor which are, therefore, well represented in crude oil related studies (National Research Council, 2003). Another research focus is anthropogenic sources where large-scale accidental oil spills like those from the Exxon Valdez collision in 1989 or the Deepwater Horizon (DWH) blowout in 2010 demand attention. Huge amounts of crude oil are released with catastrophic effects for the impacted marine habitats during such acute one-time hydrocarbon inputs, however, these events surprisingly only account for approximately 9% of the total annual emissions (Fig. 1.1). The majority of anthropogenic emissions (40% of the total annual release) occur at much lower concentrations via diffusive sources (Fig. 1.1, illustrated in grey). This often overlooked low-concentrated hydrocarbon contamination occurs via operational discharges along shipping routes, via river and runoff inputs, partially combusted aircraft fuels, and atmospheric deposition (Duran & Cravo-Laureau, 2016; González-Gaya et al., 2016; National Research Council, 2003).

A substantial share of hydrocarbons enters the marine environment as petrogenic crude oil components, yet some, especially among the diffusive sources, are also of pyrogenic or even of cosmic origin. Additionally, newer studies suggest that hydrocarbons are produced by cyanobacteria, however, they are considered to be immediately biodegraded and

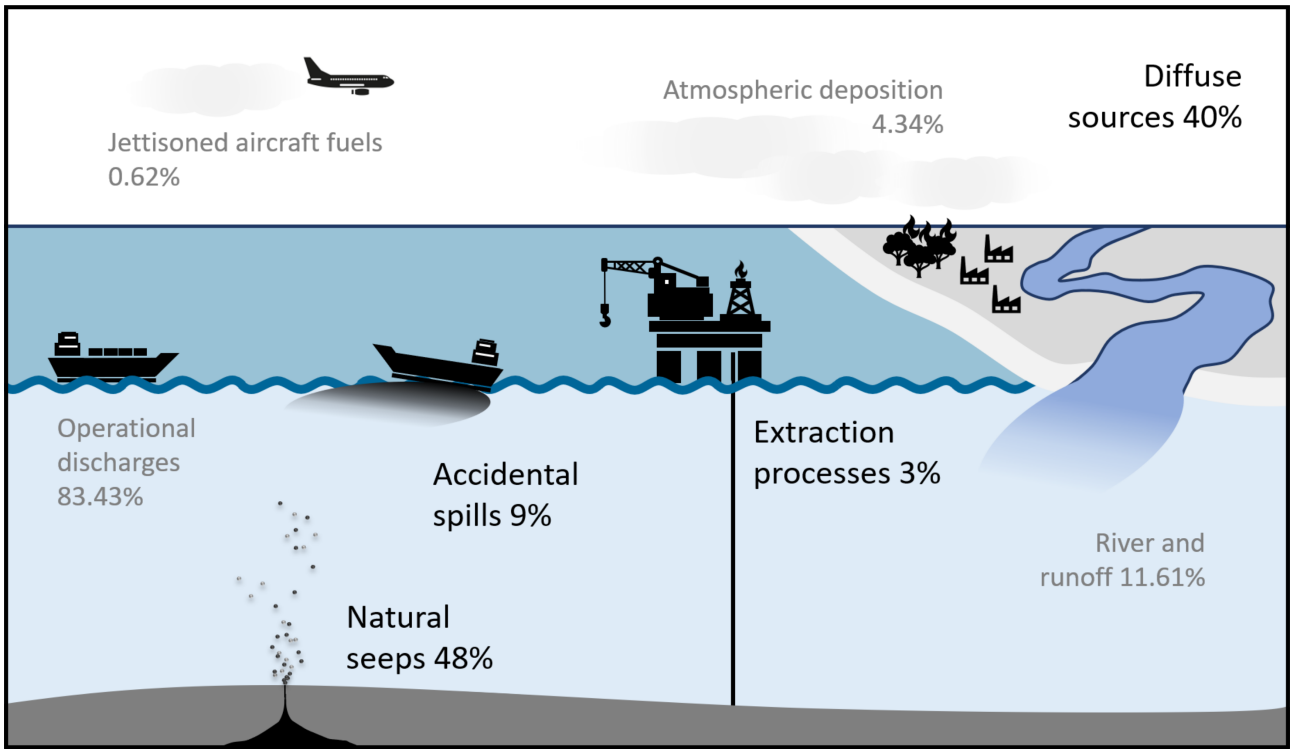

Figure 1.1: Sources of marine hydrocarbon input (National Research Council, 2003). Percentages of the total annual release (best estimate 1.3 million tons) labeled in black. The 40% attributed to diffusive sources are further divided into processes leading to low-concentrated PAH contamination and labeled in grey.

part of a currently investigated cryptic hydrocarbon cycle (Lea-Smith et al., 2015; Love et al., 2021). Low-concentrated hydrocarbon emissions are, therefore, highly variable in space and frequency which makes them difficult to predict, monitor, and study. Hence, given the substantial volume of hydrocarbons that enter marine habitats via (repeated) low-concentrated sources, it is important to determine if and how their fate differs from well-studied acute high-concentrated hydrocarbon contamination events.

1.1.2 Physico-chemical properties of (polycyclic aromatic) hydrocarbons and impacts on marine environments

Crude oil is a complex mixture of up to 20,000 petrochemical compounds (Marshall & Rodgers, 2004), and each type of oil has a different ratio of these compounds, which influences the physico-chemical properties like viscosity, volatility, and density. Typically, crude oil is classified based on density and sulfur content as light, medium, heavy, or extra heavy and sweet or sour (sulfur content $>$ 0.5%), respectively and over 80% of crude oil consists of hydrocarbons (Overton et al., 2016).

Hydrocarbons are organic carbon compounds that consist (mainly) of carbon and hydrogen and are classified depending on their chemical structure. Aliphatic hydrocarbons are linear or branched hydrocarbon chains as well as non-aromatic cyclic compounds, and make up the majority of hydrocarbons in crude oils (National Research Council, 2003; Overton et al., 2016). Aliphatic hydrocarbons can be saturated (like alkanes that are characterized by single

bonds) or unsaturated (characterized by double or triple bonds like in alkenes and alkynes) and vary in chain length and branch points. Due to their physico-chemical characteristics, they exhibit high volatility and poor water solubility, both of which decrease as the number of carbon atoms increases. Although crude oil consists mostly of aliphatic compounds, these compounds are less problematic since they were found to be less persistent in marine environments compared to polycyclic aromatic hydrocarbons (PAHs), which are far less abundant than aliphatic compounds and only make up 0.2 to 7% of crude oil components (National Research Council, 2003). PAHs consist of at least two aromatic rings with delocalized electrons stabilizing the system and are mostly planar. Due to the delocalized electrons, PAHs are relatively stable and mildly polar and their stability increases with the number of carbon atoms while polarity decreases. Therefore, low-molecular-weight PAHs have a higher water solubility than most aliphatic hydrocarbons with a similar number of carbon atoms. Ultimately, their higher water solubility increases their mobility in aqueous phases and their uptake into cells, thereby enhancing their toxicity. Combined with their resulting lipophilic properties, PAHs tend to sorb to particulate and organic matter which leads to bioaccumulation, biomagnification, and vector-bound transport of PAHs sorbed to particles (Almeda et al., 2013; Landrum et al., 2003; Meador et al., 1995). PAHs are, therefore, ubiquitous in marine environments and are considered persistent (especially with increasing molecular weight), which raises concerns for the environment as well as human health. Several PAHs and their metabolites have, moreover, been shown not only to be acutely toxic but to also have mutagenic and carcinogenic effects with some suspected of affecting the endocrine system (Kummer et al., 2008; Murawski et al., 2014; Nikolaou et al., 2009; Stading et al., 2021).

1.1.3 Fate of low-concentrated PAH inputs within marine environments

Given the hazardous potential of low-concentrated PAHs in marine environments, there is a need to understand mechanisms such as distribution, accumulation, and degradation within contaminated marine environments (Fig. 1.1). This thesis focuses on PAHs of petrogenetic origin given that operational discharges are the main source of low-concentrated hydrocarbon emissions (i.e., 83.43%, Fig. 1.1).

After emission, several immediate processes lead to a substantial decrease in the volumetric concentration without actually removing PAHs from the environment (Fig. 1.2). Given the large volume of the ocean, dilution of the emitted PAHs with uncontaminated water sets in, expedited by spreading of the slick until a minimum thickness based on dynamics between gravity and viscosity is reached (Fannelop & Waldman, 1972; M. Fingas, 2021). The observable *in situ* concentration is further reduced by volatilization of low-molecular-weight PAHs from the emitted hydrocarbon mixture, which can be further facilitated by increasing temperature, wind, and waves (Afshar-Mohajer et al., 2018; M. F. Fingas, 2004; Sabin et al., 2010). Although the evaporating chemicals are removed from the ocean, they are not removed from the overall environment, making this process important in terms of transport to and deposi-

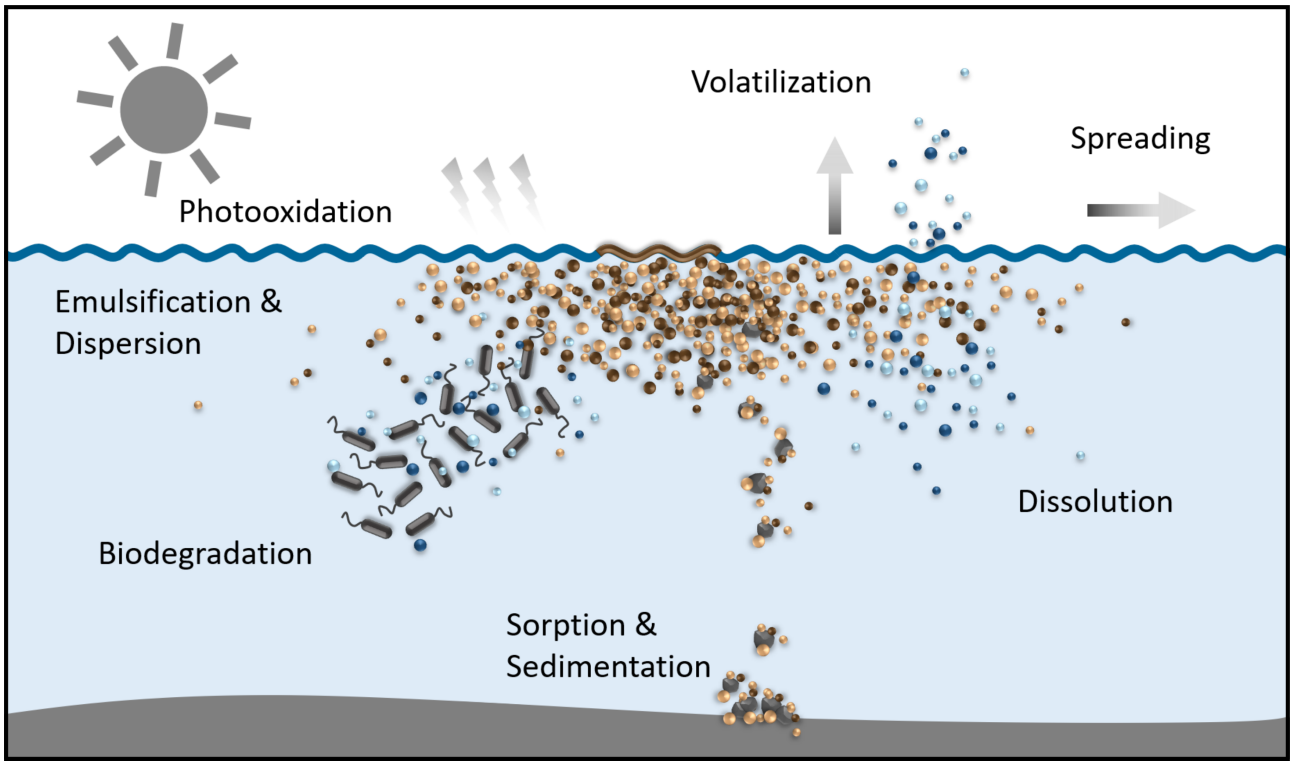

Figure 1.2: Overview of the main processes determining the fate of low-concentration PAH inputs within marine environments.

tion in pristine regions (Fernandez et al., 2021; González-Gaya et al., 2016; X. Zhang et al., 2021; Zheng et al., 2021). The remaining, less volatile compounds are subject to emulsification (water-in-oil mixture), dispersion (oil-in-water mixture), or complete dissolution in the aqueous phase, and all of those processes are promoted by currents, wind, and waves (Li et al., 2017).

Due to their hydrophobic properties PAHs sorb to organic matter and, thus, get buried in marine sediments (e.g., as part of marine oil snow (Bacosa et al., 2020; Gutierrez et al., 2018)), which acts as a main sink and reservoir of PAHs (Adhikari et al., 2016; Brion & Pelletier, 2005; Sabin et al., 2010). Due to sedimentation, burial, and low oxygen concentrations within the sediment, PAHs can be persistent in the seafloor, however, re-suspension events can also lead to desorption and remobilization of PAHs into the water column (Brion & Pelletier, 2005; Frapiccini & Marini, 2015; Latimer et al., 1999; D. A. Roberts, 2012).

Dissolution only accounts for a minor portion of the immediate mass loss of emitted PAHs since solubility in marine water is low. The dissolved fraction of PAHs, however, is frequently linked to bioavailability and, hence, its susceptibility to biodegradation (Vergeynst et al., 2019; Wodzinski & Coyle, 1974). The solubility is reliant on the physico-chemical characteristics of PAHs, as well as the temperature and ionic strength (i.e., salinity) of the aqueous phase. Dissolution of PAHs can be augmented by the presence of anthropogenic chemical dispersants or bacterial biosurfactants by enhancing emulsification, thereby increasing the water-oil surface area, and ultimately leading to a higher dissolution rate (John et al., 2016; Mulder et al., 1998; Y. Zhang et al., 1997).

The most important processes that are responsible for removing PAHs from marine environments, however, are abiotic or biotic oxidation (Atlas, 1981; González-Gaya et al., 2019). At the ocean's surface, and to a decreasing degree also within the euphotic zone, (where sunlight penetrates the water column up to a depth of 200 m) abiotic photooxidation via UV light occurs since PAHs are particularly sensitive to photooxidation (Atlas, 1981; Garrett et al., 1998; King et al., 2014). Although photooxidation may not directly result in the removal of the PAHs from the environment, the photooxidation products can alter the subsequent fate of PAHs (Thingstad & Pengerud, 1983; Ward et al., 2018). Photooxidation intermediates exhibit enhanced water solubility, and, therefore, bioavailability and toxicity, with each oxidation step, making them more susceptible to mineralization processes (Atlas, 1981; Fu et al., 2012; Islam et al., 2015; King et al., 2014; Vergeynst et al., 2019).

Similar trends regarding the degradation products can be observed for biodegradation. PAHs are rich in carbon, highly reduced, and, therefore, provide a high energy yield during oxidation. Accordingly, many microorganisms (i.e., algae, fungi, archaea, and bacteria) evolved or acquired the ability to utilize PAHs as carbon and energy sources (González-Gaya et al., 2019; Hong et al., 2008; Joye et al., 2016; Oren, 2019; Simister et al., 2015; Yakimov et al., 2007). Bacteria involved in PAH-biodegradation either perform one step of the degradation pathway as part of a PAH-degrading community or possess the complete pathway for the full degradation of one or multiple PAHs (Cui et al., 2014; Dombrowski et al., 2016; Mahjoubi et al., 2021; McGenity et al., 2012; W. Wang et al., 2018). Using genome-function relationship approaches, only recently new PAH degraders were predicted, highlighting knowledge gaps in the field of PAH biodegradation and the potential for new discoveries (for a recent phylogenetic tree of PAH-degrading strains based on genome-function relationship prediction see Huang et al., 2023). Bacterial biodegradation of PAHs is independent of light and, therefore, occurs whenever PAHs are available in marine environments — under both oxic and anoxic conditions. Marine biodegradation is, however, influenced by numerous environmental factors (for a recent review see Bacosa et al., 2022) like availability of nutrients, temperature, pressure, bioavailability of the substrate (i.e., the solubility in the given environment or the strain-specific ability to access sorbed PAHs), PAH concentrations, and availability of electron acceptors (most importantly oxygen concentrations) (Baboshin & Golovleva, 2012; Bacosa et al., 2021; Bargiela et al., 2015; Bell & Gutierrez, 2019; Duran & Cravo-Laureau, 2016; Poeton et al., 1999; Ribicic et al., 2018; Rughöft et al., 2020; Sun & Kostka, 2019; Vergeynst et al., 2019).

In the absence of oxygen, anaerobic biodegradation is important for the fate of PAHs buried in sediments. Anaerobic biodegradation is, however, limited due to lower energy yields during metabolic processes, which results in slow growth rates and relatively long mineralization times of several weeks to years for anaerobic PAH-degrading microorganisms (Meckenstock et al., 2016; Passow & Overton, 2021; Widdel & Rabus, 2001). Nonetheless, anaerobic bacteria are known to degrade PAHs under nitrate-reducing, sulfate-reducing, iron-reducing, and methanogenic conditions (Chang et al., 2006; Meckenstock et al., 2016; Rockne et al.,

2000; Rothermich et al., 2002; Z. Zhang et al., 2021). In alternating oxic-anoxic conditions, the oxic phases dominate biodegradation, for example, a 5-fold higher reduction of petroleum hydrocarbon contamination was observed during oxic phases compared to anoxic phases in oxic-anoxic cycling experiments (Karthikeyan et al., 2020). Within the oxygenated water column, aerobic degradation of PAHs is much faster and the recovery from a one-time high-input contamination event can happen within days to months depending on environmental conditions as well as the substrate(s) and the PAH-degrading organisms (Cui et al., 2014; Liu et al., 2016). Hence, aerobic microbial PAH degradation with oxygen as an electron acceptor plays a crucial role in mitigating PAH contamination, thereby safeguarding the health of marine ecosystems, marine food webs, and, ultimately, human health by reducing bioaccumulation and biomagnification.

There is, however, a knowledge gap regarding the degradation of low-concentrated PAH contamination and only a few — laboratory based — studies are available to date. Recently, seawater microcosms were incubated with different initial oil concentrations for 50 days and an additional study examined environmental flow-through incubators contaminated with two different concentrations of weathered diesel (Bacosa et al., 2021; Ryther et al., 2021). The few first results indicate, that biodegradation rates as well as abundance of hydrocarbon-degrading species are impacted by the initial hydrocarbon concentration. If low concentrations promote or inhibit hydrocarbon-degrading bacteria and biodegradation rates, however, continues to be unclear. Hence, it remains uncertain if processes and rates in biodegradation that have been studied for high-concentration scenarios can be easily extrapolated to deduce trends in biodegradation of low-concentrated PAH contamination. Predicting the fate of PAHs in a chronic pollution scenario, therefore, is difficult to date and this thesis uses a cross-disciplinary approach combining numerical modelling, laboratory-based microbiological experiments, and molecular biological tools to close this knowledge gap.

1.2 *Cycloclasticus* spp.: Microbial key-players in PAH biodegradation

1.2.1 *Cycloclasticus* spp. within PAH-contaminated marine environments

Over 300 bacterial genera have been identified to be able to degrade hydrocarbons and, of those, 11 genera contain some strains that are known to be specialized, obligate hydrocarbon degraders that almost exclusively utilize hydrocarbons as a carbon and energy source (Prince et al., 2019; Yakimov et al., 2007, 2022). From those hydrocarbonoclastic bacteria, strains within the genera *Cycloclasticus* are known for their PAH-degrading capabilities, which is reflected in the name that translates to ”ring-breaker”. The genus belongs to the class Gammaproteobacteria and is characterized by a preference for hydrocarbons as a

source of carbon and energy with a narrow substrate spectrum limited almost exclusively to PAHs such as naphthalene, phenanthrene, and pyrene (Geiselbrecht et al., 1998; Kasai et al., 2003; B. Wang et al., 2008). However, there have been a few studies indicating that some *Cycloclasticus* spp. might also degrade alkanes (Bagi et al., 2022; Gutierrez et al., 2018; Rubin-Blum et al., 2017) or can use a few organic acids as carbon and energy sources (Dyksterhouse et al., 1995).

Three ”species” (i.e., reflecting clusters of genetic similarity) within the genus *Cycloclasticus* are recognized by the List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al., 2020): *Cycloclasticus pugetii* (Dyksterhouse et al., 1995), *Cycloclasticus oligotrophus* (Y. Wang et al., 1996), and *Cycloclasticus spirillensus* (Chung & King, 2001; B. Wang et al., 2008). A fourth ”species”, *Cycloclasticus zancles*, was proposed in 2016 (Messina et al., 2016; Yakimov et al., 2016). Yet, the concept of bacterial ”species” remains controversial, with many available definitions of ”species” and some scientists question the idea altogether (Van Rossum et al., 2020). Therefore, this thesis will avoid using the ”species” definition and focus on specific *Cycloclasticus* strains instead.

Cycloclasticus spp. are marine (salt requirement $>1\%$), gram-negative, aerobic, heterotrophic, rod-shaped (0.5 μm in diameter and 1 μm in length), and exhibit chemotaxis as well as motility via a polar flagellum (Geiselbrecht, 2015; Messina et al., 2016; Staley, 2010). Although hardly detectable in uncontaminated marine samples, *Cycloclasticus* spp. grow quickly under a range of temperatures whenever PAH contamination occurs and are, therefore, considered ubiquitous (Cui et al., 2019; Gutierrez et al., 2013; Rivers et al., 2013; Rizzo et al., 2019; B. Wang et al., 2008; Yakimov et al., 2016). For example, relative abundances of sequences belonging to *Cycloclasticus* spp. were reported to be 0.2% and 64.5% in pristine and contaminated Arctic sediments, respectively, highlighting their key role in degrading petroleum hydrocarbons, particularly PAHs (Dong et al., 2015).

After a marine hydrocarbon contamination event with hydrocarbon mixtures, such as crude oil compounds or diesel, there is a typical succession of aliphatic hydrocarbon-degrading bacteria to degraders of PAHs within the microbial community. In such studies, *Cycloclasticus* spp. are regularly found to dominate the later phases of (*in situ*) microbial communities after oil spills or microcosm studies, mineralizing the abundant low-molecular-weight PAHs once aliphatic hydrocarbons have been degraded (Bagi et al., 2022; Kimes et al., 2014; Redmond & Valentine, 2012; Zhou et al., 2022). Additionally, a recent metagenomic study on oil-exposed seawater communities revealed that 25% of the investigated top 100 genes involved in PAH degradation belonged to *Cycloclasticus* spp., emphasizing their dominance particularly within the PAH-degrading fraction of the microbial community (Bagi et al., 2022). Further, *Cycloclasticus* spp. have been recognized as early colonizers of the plastisphere due to their potential plastic-degradation capabilities, which is not surprising given that common plastics are derived from fossil fuels (Denaro et al., 2020; Popovic et al., 2017; Yakimov et al., 2022). Consequently, *Cycloclasticus* spp. are found to be enriched globally in PAH-contaminated marine habitats i.e., coastal, marine, and deep-sea sediments, as well as in

the water column, or in symbiosis with mussels and sponges (Cui et al., 2008; Kimes et al., 2014; Kleindienst et al., 2016; Rubin-Blum et al., 2017). Given the environmental relevance and the substantial amount of baseline knowledge, *Cycloclasticus* spp. have been recognized as highly relevant PAH degraders that are frequently used as model organisms in pure culture studies investigating marine PAH degradation.

1.2.2 Isolated and sequenced model strains of *Cycloclasticus* spp.

Many bacterial strains within the genus *Cycloclasticus* have been isolated and are well studied, both in pure culture as well as in *in situ*-like microcosm studies. *Cycloclasticus* spp. are frequently found to be a highly abundant species in co-culture with other bacteria belonging to other hydrocarbon-degrading genera like *Novosphingobium*, *Alcanivorax*, *Thalassospira*, *Halomonas*, and *Marinobacter* when marine sediments or seawater are enriched with PAHs (Dombrowski et al., 2016; Genovese et al., 2015; B. Wang et al., 2008). In microbial communities capable of degrading hydrocarbons, synergistic interactions can be observed where individual microorganisms form symbiotic relationships with other community members. It is, therefore, likely that the PAH-biodegradation efficiency of *Cycloclasticus* spp. in such a community might be enhanced compared to the degradation efficiency of a pure culture (Laothamteep et al., 2021). In a community *Cycloclasticus* spp. could, for example, benefit from the biosurfactant production or degradation of intermediate metabolites by other community members (Dombrowski et al., 2016; Gutierrez et al., 2018).

Table 1.1: Isolated *Cycloclasticus* spp. with available genomes (NCBI database, accessed August 2023 (Schoch et al., 2020)).

Strain	Origin	Genome	G-C content	GenBank ID
C. pugetii PS-1[1]	sediment, Puget Sound	perman. draft 2 383 924 bp	41.97%	ARVU00000000
C. pugetii PY97M[2]	sediment, Yellow Sea	perman. draft 2 359 509 bp	41.92%	ASHL00000000
Cycloclasticus sp. P1[3]	deep-sea sediment, Pacific Ocean	complete 2 363 215 bp	42.00%	CP003230
Cycloclasticus sp. PY97N[4]	sediment, Yellow Sea	complete 2 430 252 bp	42.06%	CP023664
C. pugetii DSM 27168[5]	DWH oil spill, Gulf of Mexico	perman. draft 2 540 448 bp	41.57%	FQZJ00000000
C. zancles 78-ME[6]	Tar of a shipwreck site, Italy	complete 2 655 425 bp	42.00%	CP005996

[1] Dyksterhouse et al., 1995; [2]Cui et al., 2013; [3]B. Wang et al., 2008, Lai et al., 2012; [4]Cui et al., 2019; [5]Gutierrez et al., 2013; [6]Messina et al., 2016

Although the enrichment of *Cycloclasticus* spp. is straightforward using marine sediment or water where PAHs are provided as the sole source of carbon and energy, isolation of a

pure strain and its cultivation in a minimal seawater medium has been challenging (Geiselbrecht, 2015; Genovese et al., 2015; Staley, 2010). *Cycloclasticus* spp. can be more easily grown in complex media with PAHs as their primary source of carbon and energy (Genovese et al., 2015). There is still, however, a lack of understanding of the requirements needed for growing isolated *Cycloclasticus* spp. to high cell densities in minimal medium. It has been hypothesized that a lack of genes encoding for siderophores — enzymes involved in iron uptake — is one of the main causes for their lack of growth on minimal seawater media (Genovese et al., 2015). Consequently, to date, 105 metagenome-assembled genomes of uncultured *Cycloclasticus* spp. have been identified (BioSample, NCBI database, accessed September 2023 (Schoch et al., 2020)), while only six strains with sequenced genome — three of them complete genomes — have been isolated (Table 1.1). The six strains cannot be distinguished based on their 16 rRNA genes since the regions amplified by the regularly used primers (between 341 bp and 797 or 907 bp) are identical. Therefore, regardless of their isolation from distant geographic locations, the strains are considered to be very closely related to each other (Geiselbrecht et al., 1998; Staley, 2010). Genetic diver-

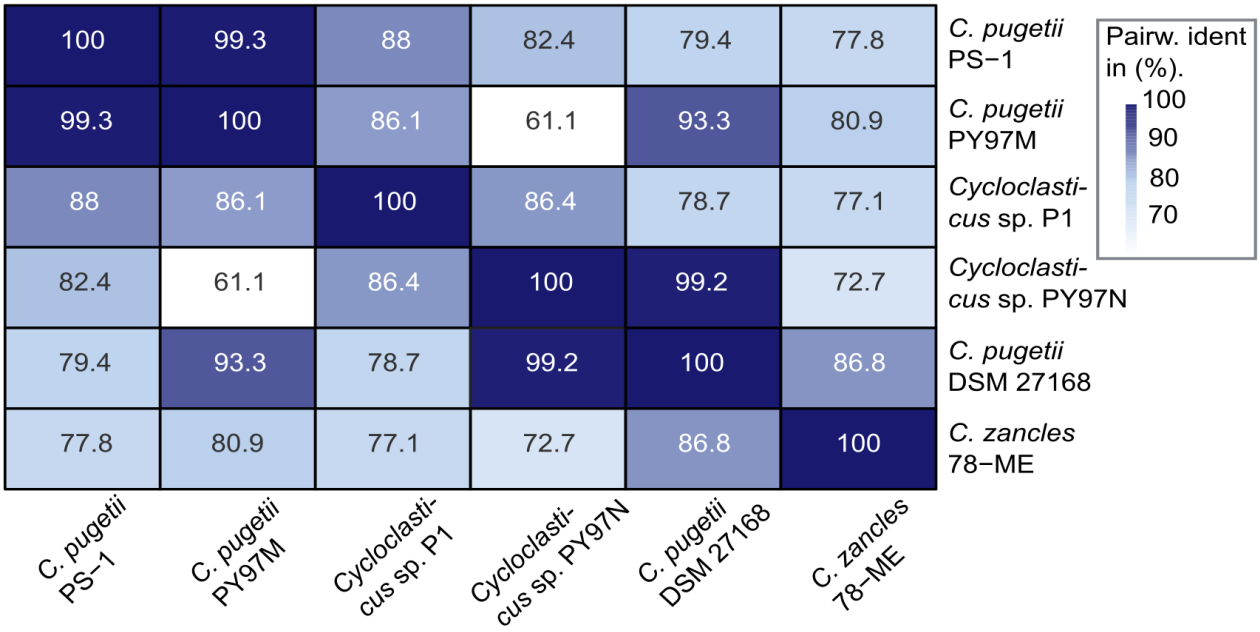

Figure 1.3: Nucleotide pairwise identity between the complete genomes of isolated *Cycloclasticus* spp. (NCBI database, accessed August 2023 (Schoch et al., 2020)).

sity between the strains can be detected using the nucleotide pairwise identity between the complete genomes (Fig. 1.3). The genetic similarity is reflected in the fact that only small differences in substrate preferences and PAH-turnover rates can be found between the isolated *Cycloclasticus* strains grown in pure culture (Geiselbrecht et al., 1998; W. Wang et al., 2018). Furthermore, the same cluster of genes involved in PAH degradation was described in two independent studies with *Cycloclasticus* sp. strain A5 (no sequenced genome available) and *Cycloclasticus* sp. strain P1 (Kasai et al., 2003; W. Wang et al., 2018), indicating *Cycloclasticus* spp. might potentially use identical enzymatic pathways to degrade PAHs

such as naphthalene and phenanthrene. Overall, the genetic similarity between *Cycloclasticus* strains suggests a close evolutionary relationship and equivalent biological functions and capabilities. Therefore, it is likely that *Cycloclasticus* spp. share metabolic degradation pathways, relevant functional genes, and respond uniformly to environmental perturbations such as PAH contamination.

1.2.3 PAH-degradation pathway & functional genes in *Cycloclasticus* spp.

The degradation of model PAHs such as naphthalene and phenanthrene by *Cycloclasticus* spp. is well studied and a complete enzymatic pathway (Fig. 1.4) was postulated based on genomic and transcriptomic analysis, enzymatic assays, and metabolite characterization (W. Wang et al., 2018, 2021). In *Cycloclasticus* spp. the degradation of model PAHs such as naphthalene and phenanthrene follows a similar pathway (Fig. 1.4). The initial and rate-limiting step is the addition of two hydroxyl groups to one of the benzene rings by a ring-hydroxylating dioxygenase (RHD; E1 and E10 for phenanthrene and naphthalene, respectively). Furthermore, the metabolite undergoes dehydrogenation by a NAD^+-dependent dehydrodiol dehydrogenase (E2 (phenanthrene) and E11 (naphthalene)), followed by *meta*-cleavage by a ring-cleavage dioxygenase (E3 - involved in both pathways) and an unstable ring-cleavage product is formed. The unstable ring-cleavage product undergoes spontaneous recyclization and is further metabolized by an isomerase (E4 - both pathways). Using hydration, a hydratase-aldolase (E5 - both pathways) breaks the carbon-carbon bond at the carbonyl group and is further oxidized via a dehydrogenase (E6 and E8 in phenanthrene and naphthalene degradation, respectively). At this point in phenanthrene degradation, the resulting 1-hydroxy-3-naphthalene aldehyde (S7) is decarboxylated by a hydroxylase (E7) and the product is funneled into the naphthalene degradation pathway. In naphthalene degradation, however, catechol is produced via oxidative decarboxylation by a salicylate 1-hydroxylase (E9) which is further converted to biological precursor molecules, such as pyruvate, that are funneled into the tricarboxylic acid (TCA) cycle.
Despite many overlapping essential steps in the naphthalene and phenanthrene degradation pathways, the enzymes involved in the initial two steps for each of the pathways are believed to be substrate-specific. Ultimately, naphthalene and phenanthrene share a downstream pathway through salicylate degradation via catechol, that is converted to pyruvate and funneled into the TCA cycle for energy generation.
The genes encoding for the enzymes involved in PAH degradation are used as functional markers for bacterial PAH degradation in several PAH degraders and multiple habitats such as soil, freshwater, and marine sediments (Cebron et al., 2008; Iwai et al., 2011; Liang et al., 2019; Marcos et al., 2012; Meynet et al., 2015). Given that RHDs initialize the first, rate-limiting step and are thought to be more substrate-specific, it is not surprising that most research has focused on those characteristic PAH-degradation enzymes. Genes encoding

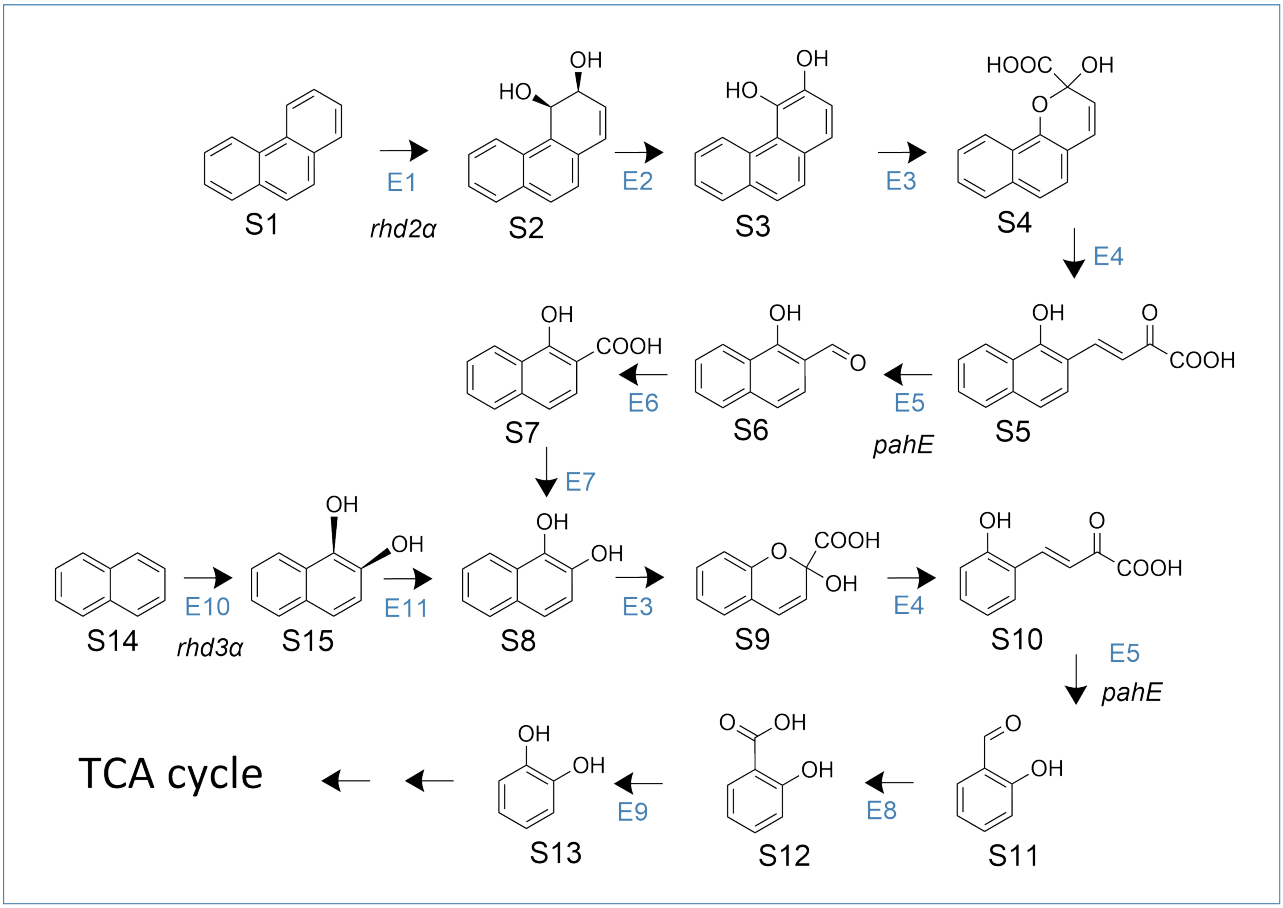

Figure 1.4: Degradation pathway for naphthalene and phenanthrene in *Cycloclasticus* spp. (W. Wang et al., 2018). Chemical designations: S1, phenanthrene; S2, *cis*-3,4-phenanthrenedihydrodiol; S3, 3,4-dihydroxyphenanthrene; S4, 2-hydroxy-2H-benzo(h)chromene-2-carboxylic acid; S5, *trans*-4-(1'-hydroxynapth-2-yl)-2-oxobut-3-enoic acid; S6, 1-hydroxy-2-naphthaldehyde; S7, 1-hydroxy-2-naphthoic acid; S8, 1,2-dihydroxynaphthalene; S9, 2-hydroxy-2H-chromene-2-carboxylic acid; S10, *trans*-o-hydroxybenzylidenepyruvic acid; S11, salicylaldehyde; S12, salicylic acid; S13, catechol; S14, naphthalene; S15, (1R,2S)*cis*-l,2-naphthalenedihydrodiol. Enzyme designations (in blue) with functional marker genes investigated in Vogel et al. 2023b: E1, phenanthrene ring- hxdroxylating dioxygenase (*rhd2a*); E2, dihydrodiol dehydrogenase; E3, ring-cleavage dioxygenase; E4, isomerase; E5, hydratase-aldolase (*pahE*); E6, aldehyde dehydrogenase; E7, 1-hydroxy-2-naphthoate hydroxylase; E8, salicylaldehyde dehydrogenase; E9, salicylate 1-hydroxylase; E10, naphthalene ring-hydroxylating dioxygenase (*rhd3a*); E11, NAD^+-dependent *cis*-1,2-naphthalenedihydrodiol dehydrogenase

for RHDs, like *rhd3*α and *rhd2*α (Fig. 1.4) are, therefore, potentially useful to determine the substrate preferences of PAH-degrading bacteria based on their genome and track PAH-degradation activity through transcription-based methods.

Several enzymes used during naphthalene degradation are also believed to be involved in phenanthrene degradation like the ring-cleavage dioxygenase (E3, 3^{rd} step), isomerase (E4, 4^{th} step), and hydratase-aldolase (E5, 5^{th} step). The genes encoding for those enzymes, like *pahE* which encodes for a hydratase-aldolase are, therefore, interesting as functional marker genes involved in general PAH degradation (Liang et al., 2019).

Given the substantial PAH-degradation capability, the particular adaption to marine environments, and the genetic understanding that can be used to investigate PAH degradation more thoroughly, one strain (PS-1) from this keystone genus, *Cycloclasticus*, was selected as a

model organism for the presented thesis.

1.3 Quantification of PAH-biodegradation activity

To determine biodegradation activity in marine environments, the microbial degradation of hydrocarbon mixtures or single model PAHs is followed while quantifying cell numbers or activity. Standardized routine methods that can be used to calculate *in situ* PAH-degradation rates are, however, largely lacking. Therefore, PAH-degradation rates are quantified in the laboratory and often estimated as mean values over longer incubation times (Bacosa et al., 2021; Nolvak et al., 2021). Commonly, *in situ*-like microcosms with autochthonous microbial communities or more controlled batch experiments with model organisms are used, depending on the investigated research questions. The applied methods for determining PAH degradation include time-series hydrocarbon quantification in biotic treatments via liquid chromatography or gas chromatography coupled to mass spectrometry or flame-ionisation detection (Wilkes, 2010). Meanwhile, the microbial activity is commonly estimated through biomass increase over time determined via optical-density measurements, microscopy, or molecular tools (e.g., quantitative polymerase chain reaction (qPCR)-based cell-number quantification). Alternatively, the PAH biodegradation rate in mg L^{-1} $time^{-1}$) can be measured by using ^{14}C-labelled PAHs in radio-tracer assays (Kleindienst et al., 2015). Aliquots of the investigated microbial community or culture are incubated with ^{14}C-labelled PAHs for a defined period of time and the ^{14}C-CO_2 produced through mineralization is determined by quantifying radioactivity. Using ^{14}C-PAH-oxidation assays, however, does come with limitations like accounting for the ”dilution” of the radioactive signal with CO_2 from mineralization of unlabelled PAHs or requiring initial knowledge about the PAHs-degradation activity in order to choose an appropriate incubation period. Furthermore, working with radioactive substances requires specialized facilities and safety regulations. Quantifying PAH-degradation rates through canonical methods is challenging to conduct *in situ* — especially at low PAH concentrations or in a high-throughput manner — leading to a gap in the predictive understanding of the fate of environmental PAHs. Thus, alternative methods are needed to enhance monitoring of microbial PAH-degradation activity in marine environments.

The ratio of mRNA to DNA of characteristic functional genes — i.e., the dimensionless so-called transcript-to-gene (TtG) ratio — could provide an *in situ* method for estimating the rate of PAH-degradation and, therefore, serve as a cultivation-independent method that potentially correlates with per-cell PAH-degradation rates (Baelum et al., 2008; Bagi et al., 2022; Brow et al., 2013; Knapik et al., 2020; Tentori & Richardson, 2020). Correcting the gene transcription against a potential overestimation of activity due to growth, the TtG ratio can recognize real, per cell change — or the lack thereof — in microbial PAH-degradation activity (Baelum et al., 2008; Brow et al., 2013; Tentori & Richardson, 2020). It remains, however, largely unknown which functional PAH-related genes are suitable target-gene candidates

and if the TtG ratio is a useful proxy for PAH-biodegradation activity, especially at low concentrations.

Moreover, incorporating data from existing studies on PAH degradation at high concentrations and developing models that simulate microbial PAH degradation could begin to close the knowledge gap regarding marine low-dosage PAH pollution. Although field and laboratory-based studies are not replaceable in the understanding of environmentally relevant processes, they are limited in temporal and spatial resolution, as well as in the number of experiments. Using conceptual models that represent our current understanding of interconnected processes, driving parameters related to microbial PAH degradation, and ultimately, translating our experimental knowledge into mathematical equations can, therefore, be used as an additional tool for microbiological experimental research (Brimo et al., 2016; Soulas & Lagacherie, 2001). Hence, based on our knowledge on high-concentrated PAH contamination, a variety of initial conditions — like PAH concentrations — can be tested and simulated results can identify highly relevant experimental setups for future studies. Thus, using numerical modelling can ultimately help to predict the fate, as well as PAH-degradation activity of unexplored low-dosage PAH contamination within marine environments.

2. Research aims

2.1 Problem statement and dissertation outline

Biodegradation of high hydrocarbon and PAH loads is a well-studied attenuation process within marine environments and has received a particularly high level of attention following the Deepwater Horizon blowout in 2010 (Head et al., 2006; Joye et al., 2016; Kleindienst & Knittel, 2020; Mason et al., 2012). Given the substantial volume of PAHs entering marine habitats via (repeated) low-concentrated emissions, there is a crucial need to understand low-concentration PAH biodegradation. To date, knowledge of the degradation dynamics of repeated low-dosage PAH contamination is lacking with only a few studies that have investigated the fate of PAHs in a chronically polluted marine water column (Bacosa et al., 2021; Krolicka et al., 2019; Mostafa et al., 2019; Ryther et al., 2021).

The impact of dosage concentration and frequency of PAH input on biodegradation dynamics and the recovery of the system is still unknown. However, research on other contaminants (e.g., herbicides) suggests that frequency and dosage of releases highly influence the biodegradation efficiency of contaminants (Baelum et al., 2008; Lancaster et al., 2010). These questions need to be addressed in the field of PAH biodegradation, since closing these knowledge gaps is very relevant for risk assessment and contamination management, particularly for recurring pulse-like PAH-pollution events.

Currently, there is a lack of methods that can follow PAH-degradation activity under *in situ*(-like) conditions. By targeting functional genes involved in PAH degradation, the TtG ratio could be a promising culture-independent measure to quantify biodegradation activity (Baelum et al., 2008; Bagi et al., 2022; Brow et al., 2013; Knapik et al., 2020; Tentori & Richardson, 2020). However, it remains unclear which PAH-degradation genes are satisfactory target genes and how the TtG ratio correlates with biodegradation rates. It is also unknown how varying PAH loads influence the transcription of functional genes involved in PAH degradation.

To address these questions, the work presented in this thesis used an interdisciplinary approach combining methods from environmental modelling, (molecular) microbiology, and petrogenic chemistry. A combination of numerical modelling, qPCR-based assays, and RNA sequencing aimed at improving our understanding of biodegradation activity of *Cycloclasticus* spp. from an intracellular to an environmental system level. Consequently, in understanding

the involved processes on a small scale, this thesis provides the foundation for expanding our knowledge of hydrocarbon biodegradation dynamics and therefore the fate of crude oil in marine environments. The objectives addressed in the presented thesis are stated below:

2.2 Research questions addressed in the thesis

- (i) Can we track PAH-degradation activity using TtG ratios of functional marker genes? Addressed in Vogel et al., 2023b and Vogel et al., 2024.
- (ii) Does the concentration of PAH input influence the microbial degradation performance? Addressed in Vogel et al., 2023a and Vogel et al., 2024.
- (iii) How does transcription change under varying PAH concentrations and does this affect the use of TtG ratios? Addressed Vogel et al., 2023b and in Vogel et al., 2024.

3. Results and general discussion

3.1 Evaluating the PAH-degradation activity of *Cycloclasticus* spp. using functional genes

TtG ratios provide a promising approach to track PAH degradation rates during the active growth phase of PAH-degrading bacteria by quantifying the gene and transcript numbers of functional genes involved in PAH degradation. Given this, one aim of this thesis was to develop a molecular tool to track PAH-degradation activity in *Cycloclasticus* spp. that could be subsequently applied in the environment. However, the unexpected results presented in Vogel et al., 2023b showed a lack of transcriptional response of the investigated target genes during active PAH degradation. Essentially, the TtG ratios of *rhd2*α, *rhd3*α, and *pahE* did not correlate with the degradation performance of *Cycloclasticus pugetii* strain PS-1 in the pure culture experiments incubated with naphthalene, phenanthrene, a mixture of both PAHs, and a no-PAH carbon source. For instance, the TtG ratios of *rhd2*α, *rhd3*α, and *pahE* in naphthalene-only treatments did not change significantly between day 4 and 7 while the degradation rate decreased from 63.6 to 5.65 mg L^{-1} day^{-1} during the same time frame and 86.5% of the available naphthalene was degraded between day 3 and 6. Similar trends were observed in Arctic microcosm experiments where the TtG ratio of the target gene (of the three target genes, only *rhd2*α was found in the samples) was quantified for *Cycloclasticus* spp. from natural seawater communities incubated with either a low-repeated or medium one-time input from a complex hydrocarbon source (water-accumulated fraction (WAF) hydrocarbons). The TtG ratio of *rhd2*α, with a mean of $4.61 \times 10^{-1} \pm 1.01 \times 10^{-1}$ and $3.63 \times 10^{-1} \pm 2.12 \times 10^{-1}$ for low-WAF and medium-WAF microcosms, respectively, was found to be similar across time points and experimental conditions, suggesting that neither incubation time nor WAF concentration had any significant influence on the expression of the target gene. Despite the investigated marker genes being well studied and suggested as biomarkers for PAH degradation, the presented work demonstrates that using their TtG ratios in *Cycloclasticus* spp. is not suitable for quantifying PAH-degradation activity. However, the developed primers and qPCR methods can be used to identify the abundance of *Cycloclasticus* spp. within microbial communities and, hence, detecting *rhd2*α, *rhd3*α, or *pahE* in environmental DNA or RNA likely indicates the presence — although not the PAH-degradation activity — of this important PAH degrader.

Nonetheless, the results presented in Vogel et al., 2024, suggest there could be an alternative suitable target-gene candidate for *Cycloclasticus pugetii* strain PS-1 that could potentially be tracked with a qPCR assay and that might reflect naphthalene-degradation activity. The genes CYCPU RS0105800 (encoding for a hydratase aldolase, similar to *pahE*), *rhdPS1*α, and *rhdPS1*β (encoding for the large and small subunits of an RHD, respectively), showed high transcription levels under naphthalene-containing conditions and when normalized to the gene copy number (TtG ratio) demonstrated changes over time that reflected the degradation of naphthalene. Particularly, *rhdPS1*α demonstrated a very high expression ($\mathrm{TPM}_{\mathrm{mean}}$ within the 95th percentile) and statistically significant upregulation compared to the no-PAH control when the organism was actively degrading naphthalene 2 hours after inoculation with high (100 mg L^{-1}) and low (30 mg L^{-1}) naphthalene as well as 2 hours after receiving a repeated 30 mg L^{-1} pulse. Less obvious, but similar, was the expression pattern of the hydratase aldolase encoding gene CYCPU RS0105800. Significant downregulation in the high-NAP treatments during naphthalene starvation (168h) combined with a high (although not significantly high) transcription after the pulse, hinted at a positive transcriptional response to naphthalene-degradation activity. Therefore, *rhdPS1*α, is a promising target gene to quantify naphthalene-degradation activity by using the TtG ratio. CYCPU RS0105800, on the other hand, could be a *pahE*-like target gene sensitive to general PAH degradation, as previously suggested by Liang et al., 2019. Further studies should develop qPCR primers and methods in order to follow this promising lead. Further, when detecting transcripts of *rhdPS1*α in metatranscriptomes, it is very likely that the respective *Cycloclasticus* spp. are actively degrading naphthalene. However, further research is necessary to test the expression of *rhdPS1*α with different PAH substrates and to confirm the high expression during naphthalene degradation of the organism within an environmental community.

The same work also showed that *Cycloclasticus* spp. have more than one of these key enzymes (RHDs) and that the expression of their respective genes does not follow a uniform trend (Vogel et al., 2024). *Cycloclasticus pugetii* strain PS-1 notably transcribed 14 genes (TPM was within the 90^{th} percentile in at least one naphthalene-containing treatment) which were encoding for subunits of at least 6 different RHDs. 10 of these RHD-encoding genes were upregulated in the presence (8) or absence (3) of naphthalene, 3 of them substrate-independently expressed (expressed at the same level regardless of the concentration of naphthalene, including in the no-PAH control) and one did not follow a clear transcription trend. These novel results highlight a current knowledge gap in our understanding of the functional marker-gene expression. In order to select a suitable biomarker for the quantification of PAH-degradation activity — e.g., by using TtG ratios — detailed information about the transcriptional behavior of the potential genes of interest in correspondence to changes in PAH concentration is required.

In conclusion, the following questions remain unaddressed in the presented thesis and are discussed in the last section of this chapter:

- Is there genetic redundancy in key-functional genes of other PAH-degrading bacteria?
- How specific are RHDs in terms of PAH usage and is there a way to predict their affinity to specific PAHs from *in silico* genome analysis?
- Are there differences in the transcription of PAH-related genes on a single-cell level and, if so, what controls this heterogeneous expression within the population?

3.2 PAH biodegradation in *Cycloclasticus* spp. is influenced by PAH concentration

PAH concentrations vary widely in the environment, depending on the magnitude and frequency of their source, and this, in turn, impacts the PAH-biodegradation rate. One factor governing biodegradation at lower PAH concentrations was the dissolution kinetics of the substrate (Vogel et al., 2023a). Using a process-based numerical model to predict the dynamics of biomass growth and PAH concentrations of simulated degradation experiments, model results showed that the gradient between pure and dissolved PAH was the main driving force for the degradation kinetics in a closed batch system for the investigated concentration range below slick formation ($\mathrm{C_{slick}}$, Fig. 3.1) (Vogel et al., 2023a). Following Fick's first law, a higher difference in concentration between the pure phase reservoir and the dissolved PAH led to faster dissolution kinetics, given that the pure phase reservoir was available as crystals and not in the form of a slick. Assuming that only the dissolved fraction of the PAH is bioavailable and given the low solubility of PAHs in seawater (e.g., 28.94 mg L^{-1} for naphthalene), the dissolution kinetics determine the rate of substrate resupply for the bacteria. *Cycloclasticus* spp. do not produce biosurfactants, which can facilitate the dispersion and acquisition of hydrocarbons, therefore, their growth is inhibited at lower naphthalene concentrations when the gradient between the (low-concentrated) pure and the dissolved PAH is small. This is reflected in a slower generation time (3.81 days) and higher naphthalene residuals (16.5%) in simulated batch cultures receiving repeated low-dosages of naphthalene (S4) compared to simulated cultures with a one-time high-input (S1, generation time 0.65 days and naphthalene residual 0.3%) (Vogel et al., 2023a). Similarly, the *in vitro* results presented in Vogel et al., 2023b demonstrate how, in single-compound treatments, 200 mg L^{-1} of naphthalene were consumed faster and completely in 12 days, while 200 mg L^{-1} of phenanthrene were only degraded to approx. 50% in the same time frame. In the mixed-PAH treatments (100 mg L^{-1} naphthalene and phenanthrene) naphthalene degraded rapidly whereas phenanthrene degradation only occurred after naphthalene was degraded. Both examples illustrate how the degradation rate was limited by dissolution kinetics and higher bioavailability (dissolution) led to faster degradation and a preference for the more soluble naphthalene over phenanthrene.

A lower substrate availability also restricted microbial growth and the resulting overall PAH

degradation was, thus, constrained due to a slowed increase in PAH-degrading organisms. This is the second factor that governs PAH biodegradation at lower PAH concentrations. The predictions from the process-based numerical model in Vogel et al., 2023a revealed that, in the simulated experiments, the biomass concentration in each of the four scenarios, S1 through S4, was in the same order of magnitude after 10 days. However, in S1 (with an initial one-time dosage of 144.8 mg L^{-1}) 99.7% of the naphthalene was degraded and the simulated bacteria eventually reached stationary phase after seven days with a generation time of 0.65 days. Conversely, in S4 (10 pulses of 14.48 mg L^{-1} during the same time frame), the simulated batch culture grew linearly with a generation time of 3.81 days, failing to reach the stationary phase by the end of the simulation. Consequently, in S4, only 83.5% of the added naphthalene was degraded, leading to an accumulation of naphthalene by the end of the simulated incubation. When comparing the growth patterns and degradation dynamics over all the simulated scenarios it was shown that pulsing dosage and frequency strongly affected the overall degradation – adding the limited growth of PAH-degrading bacteria as a second layer to the restrictions due to dissolution kinetics. Conclusively, the results indicate that the biomass increase of PAH-degrading organisms by itself cannot be used as a reliable indicator of PAH degradation. This needs to be considered in further studies when comparing degradation performances under different concentrations. One way to purposefully eliminate growth as an influencing factor from the study when comparing e.g., transcription in treatments with different substrate concentrations is by using cell suspension experiments like in the presented RNA sequencing study (Vogel et al., 2024).

Complete transcript quantification and analyses of *Cycloclasticus* spp. under varying naphthalene concentrations (Vogel et al., 2024) identified two putative enzymatic systems that respond differently to lower or higher concentrations of PAH. Hence, the presented results indicate that due to those two enzymatic systems the available PAH concentration can further affect biodegradation through the activation — or lack thereof — of the ”rapid response” system. Considering that published environmental PAH concentrations are in the ng to µg L^{-1} range *Cycloclasticus* spp. might commonly operate with the proposed “background” system of enzymes, for which the genes are substrate-independently expressed (Vogel et al., 2024). Presumably, at a yet-to-be-determined threshold concentration of PAHs ($\mathrm{C_{thresh}}$, Fig. 3.1), the “rapid response” system with enzymes encoded by PAH-dependently expressed genes is activated. Given that the solubility in seawater is the maximum dissolved — and therefore bioavailable — concentration the organism could have adapted to, the threshold concentration is likely to be below the maximum solubility concentration of the PAH ($\mathrm{C_{sol}}$, Fig. 3.1). The transcription of the naphthalene-dependently expressed genes in the investigated strain PS-1 was, indeed, high in all 2-hour naphthalene treatments (high-NAP, low-NAP, and pulse-NAP), although, low-NAP and pulse NAP contained concentrations only slightly above the maximum solubility. Activation of the ”rapid response” system might, thus, be a mechanism to promote biodegradation and, thereby, cell growth in a PAH concentration range when dissolution kinetics are still limiting and competition between PAH-degrading populations is

high.
Therefore, in the concentration range between no PAHs and $\mathrm{C}_{\mathrm{slick}}$ a higher concentration might enhance the biodegradation activity by increasing the intracellular concentrations of enzymes involved in the biochemical pathway in addition to the favorable dissolution kinetics and the promotion of bacterial growth as illustrated in Fig. 3.1.
The presented results agree with results from the one other study that investigated the influence of PAH concentration on biodegradation rates at low PAH loads (Bacosa et al., 2020). The investigated ranges of PAH concentrations, which are responsible for enhancing biodegradation, in both the previous publication (Bacosa et al., 2020) and this thesis are all below the concentration at which hydrocarbons would form a slick ($\mathrm{C}_{\mathrm{slick}}$, Fig. 3.1) and, therefore, are representative of diffusive hydrocarbon input. Conversely, in an environmental high-pollution scenario in which a huge volume of a hydrocarbon mixture was added e.g., due to an accidental oil spill, $\mathrm{C}_{\mathrm{slick}}$ — depending on the composition and the viscosity of the hydrocarbon mixture — would most likely be reached and exceeded (Smeeth et al., 1996; Tadros, 2017). Slick formation would increase the oil/water interfacial tension while decreasing the specific surface area of the hydrocarbon mixture (Dapčević Hadnađev et al., 2013; Overton et al., 2016). Consequently, at such high hydrocarbon concentrations, the dissolution rate would decrease, the availability of the substrate would be limited, the biodegradation rate would decrease slightly, and further increase in biomass would be inhibited. Therefore, considering the physico-chemical properties of marine pollutants, is imperative to consider potential hydrocarbon concentrations when assessing the risk of PAH and oil contamination and the expected biodegradation activity.
Conclusively, the work of the presented thesis demonstrates how PAH concentration — and consequential substrate availability — is an important environmental condition that can influence the PAH-degradation rate considerably and should be accounted for in future studies. The following questions were left unanswered and are addressed in the final section of this chapter:

- Is the modelled impact of dosage concentration and frequency representative of real-world responses?
- Is there a concentration threshold at which the “rapid response” system is activated and is there a way to predict it e.g., from physico-chemical properties of the PAH?

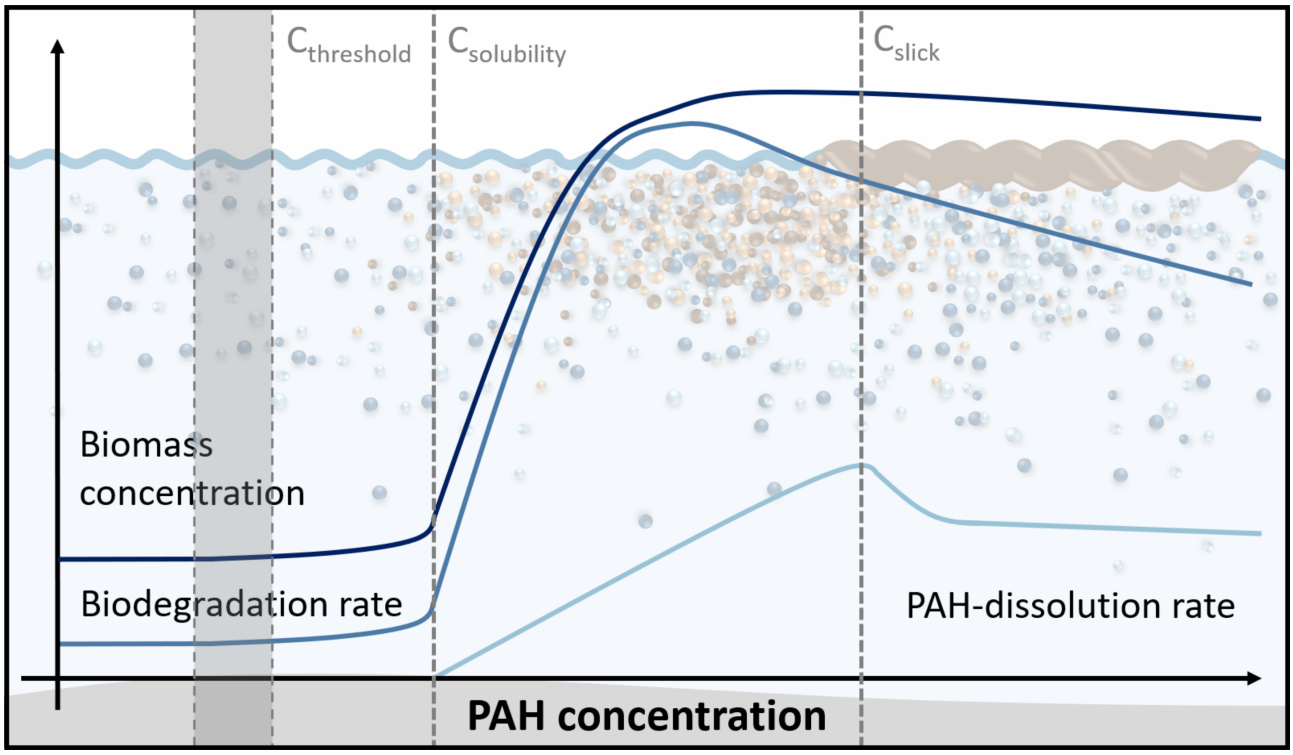

Figure 3.1: Biomass concentration, biodegradation, and PAH-dissolution rate depend on PAH concentration in marine environments. Particularly, three key parameters govern the degradation dynamics: the maximal solubility ($\mathrm{C_{sol}}$), the concentration at which the provided hydrocarbons form a slick ($\mathrm{C_{slick}}$), and the proposed threshold concentration ($\mathrm{C_{thresh}}$, yet-to-be determined and therefore illustrated as a concentration range) at which the ”rapid response” enzymatic system of specialized PAH degraders is activated.

3.3 Environmental implications

The results of the presented thesis are highly relevant for understanding impacts on marine PAH-biodegradation activity on several levels and, hence, how the fate of oil in the ocean can be monitored and predicted in the future. One of the main environmental implications is that functional redundancy and, therefore, the availability of the two proposed enzymatic systems could be a sign of increased adaptation in highly specialized PAH degraders (Vogel et al., 2024). The identification of 15 highly expressed and, therefore, likely relevant genes for at least six different RHDs — all enzymes that are believed to initiate PAH degradation — was an unexpected finding. Further examination of the transcriptome revealed that strain PS-1 was transcribing multiple genes for multiple enzymes for each step of the pathway. By categorizing the genes based on the transcriptional response to the provided PAH two enzymatic systems were proposed: one in which the encoding genes were transcribed substrate-independently resulting in ”background” PAH degradation, and a second system in which the encoded genes were transcribed in reaction to naphthalene, thereby enabling a ”rapid response” after PAH exposure. Genes of both enzymatic systems were identified in the model organism *Cycloclasticus pugetii* strain PS-1 which might emphasize the high level of adaptation to a PAH-degradation lifestyle (Yakimov et al., 2007). The genetic flexibility might allow highly specialized PAH degraders like *Cycloclasticus* spp. to adapt to changing

PAH dosages by activating the “rapid response” system when faced with huge quantities of PAH. By increasing the degradation activity rapidly, such well-adapted PAH degraders might, therefore, be able to outcompete other PAH-degrading bacteria that can only conduct one or a smaller subset of the steps in the PAH-degradation pathway (Sieradzki et al., 2021). Hence, genetic redundancy could be an evolutionary benefit in a specialized PAH degrader and a sign of a high level of adaptation to both chronic traces of PAH input as well as recurring high-input PAH pollution. Ultimately, identifying genetic redundancy in organisms within an environmental community could indicate a high level of adaption to PAH input, and therefore a faster environmental recovery from acute high-input contamination.

Additionally, the results presented in this thesis start to close the knowledge gap on how prediction of PAH-degradation activity in environmental communities could be possible using genomic and transcriptomic methods (Vogel et al., 2024, 2023b). Although there is currently no *in situ* method to quantify the PAH-degradation activity of an environmental community, the presented results may provide a starting point to develop such a valuable tool. If the qPCR-based TtG ratio of PAH-dependently expressed genes like *rhdPS1*α are proven to correlate with the degradation activity of *Cycloclasticus* spp. not only in pure cultures but also within environmental communities, this would be a major step in monitoring *in situ* PAH-degradation activity. This is especially valid given how *Cycloclasticus* spp. are often dominant members of PAH-degrading communities. This would also be a step up from the previous practice of DNA-based targeting of *Cycloclasticus* spp. abundance as a measure for PAH biodegradation since *in situ* degradation activity rather than degradation potential would be investigated. Given the similar final biomass concentrations from simulated *Cycloclasticus* spp. batch cultures with very different naphthalene-degradation activities in this thesis (S1 vs. S4 in Vogel et al., 2023a), using such true measures of biodegradation activity are imperative for truly monitoring bioremediation processes.

Furthermore, with the increasing magnitude of environmental metagenomic and metatranscriptomic studies and our capability for bioinformatic data mining, it is important to consider what the detection of genes and transcripts from genes within the $\mathrm{NAP}_{\mathrm{pos}}$ and $\mathrm{NAP}_{\mathrm{indep}}$ categories would imply for the respective microbial communities and environments. Identifying organisms with genes from the $\mathrm{NAP}_{\mathrm{indep}}$ category within a metagenome marks those community members as PAH-degrading organisms. Genes encoding for enzymes within the proposed “background” system such as *rhd3*α, or *pahE* could be — given that the genes are truly PAH-pathway specific — genomic markers for PAH-degrading bacteria as previously suggested (Dionisi et al., 2011; Liang et al., 2019). The transcription of constitutively or PAH-independently expressed genes, however, does not imply that the community members are actively degrading PAHs, but rather indicates a general metabolic activity. For assessing the PAH-biodegradation performance of PAH-degrading community members within a metatranscriptome, it would be necessary to evaluate the transcription of (a set of) PAH-dependently expressed target genes (e.g., including *rhdPS1*α and others from the $\mathrm{NAP}_{\mathrm{pos}}$ category). In that case, normalization of the metatranscriptomic data by quantitative (DNA-based) mea-

sures of a marker gene (i.e., 16S rRNA gene or even PAH-degradation gene) or absolute cell counts would eliminate changes in transcription due to cell numbers (Y. Zhang et al., 2021) and, thus, provides a metatranscriptomic-wide TtG ratio that correlates well with degradation activity. Ultimately, genes from the $\mathrm{NAP}_{\mathrm{pos}}$ category could potentially not only identify PAH-degrading organisms (on a DNA basis) but could (on an RNA basis) also be true markers for PAH-degradation activity. Further, identifying organisms that transcribe genes encoding for enzymes from both categories in a metatranscriptome is a sign of genetic redundancy. The respective communities are likely to show a high level of adaption to PAH input and, hence, faster environmental recovery from acute high-input contamination. Nevertheless, there are still many open questions on how transcription of PAH-degrading genes relates to biodegradation activity. For now, metagenomic and -transcriptomic studies that try to answer that question must always be accompanied by biochemical data to monitor the decrease in PAH concentrations and prove biodegradation.

Another main finding of this thesis' research was that pristine environments are potentially more vulnerable to even low-level PAH contamination since degradation of trace amounts was slowed down since no PAH-degrading community was established (Vogel et al., 2023a). Comparing the predictions scenarios with different pulsing frequencies from the numerical model in Vogel et al., 2023a demonstrates how less frequently applied low-concentration hydrocarbon pulses could lead to a long-term accumulation of PAHs. This contradicted the nearly complete degradation that would have been expected from extrapolating the one-time high-input scenario. Such accumulation of PAHs has indeed been described for several pristine areas like alpine lakes or mountain areas (Carrera et al., 2001; Vilanova et al., 2001). Conclusively, the presented results indicate that system recovery of pristine marine environments takes longer for repeated low-concentration contamination events. Two driving mechanisms for this slow recovery could be identified. Firstly, lower pulsed PAH input leads to kinetic inhibition of dissolution and consequently to less bioavailable substrate for PAH-degrading bacteria. In turn, PAH-degrading microorganisms are prevented from growing to high cell densities which, ultimately, leads to less biodegradation activity and a retardation of the system's recovery. The described effect is amplified when the time between dosages is prolonged as illustrated by comparing the generation times and incubation times of S5 and S6 Vogel et al., 2023a. A second parameter inhibiting a fast recovery of pristine environments might be the lack of adaptation of the native bacterial community to PAH contamination. It is not expected to find specialized PAH-degrading bacteria thriving in truly PAH-free environments but rather to have a potentially dormant seed population of PAH degraders (Gibbons et al., 2013; Joye et al., 2016). Such a PAH-degrading seed population, however, might be less adapted to PAH degradation than an active PAH-degrading community from a contaminated environment, e.g., the Gulf of Mexico. Although this is highly speculative, the identified absence of canonical PAH genes (i.e, *rhd3*α and *pahE*) in *Cycloclasticus* spp. from Arctic seawater could be a first piece of evidence (Vogel et al., 2023b). Hypothetically, *Cycloclasticus* spp. from pristine environments could, therefore, be less adapted towards PAH

degradation than a *Cycloclasticus* spp. from an environment that is regularly confronted with PAH-input and might not even possess the proposed genetic redundancy and resulting in two enzymatic systems. A potential lack of adaptation within pristine PAH-degrading community members might, hence, further inhibit a quick system recovery from PAH contamination, not only because of the limited availability but also due to comparably less efficient usages of the growth substrate. Given that the major part of anthropogenic PAH contamination in marine environments occur at low concentration and on a relatively repeated basis, the environmental relevance of the presented results is significant. The predicted PAH-degradation dynamics within pristine environments should, therefore, be considered when assessing how pristine environments like the Arctic will, in the future, be used for oil exploration and shipping lanes.

Ultimately, the following questions remain unresolved in the presented thesis and are addressed in the last section of the current chapter:

- Is there genetic redundancy in key-functional genes of other PAH-degrading bacteria and how can we differentiate between genes from the two proposed enzymatic systems?
- Are PAH degrading genes core or accessory genes of *Cycloclasticus* spp. and are, particularly, the genes from the two proposed enzymatic systems core or accessory genes?

3.4 Open questions and outlook

The results presented in this thesis start to close our knowledge gaps concerning the impact of PAH concentration on the transcription of functional marker genes and the resulting biodegradation activity. However, the results also open up new questions and help to identify necessary future studies. The last section of this chapter will, therefore, first review the consequential steps that should be investigated before the remaining open questions are discussed in detail.

Collectively, further work is required to characterize the key genes involved in PAH degradation by *Cycloclasticus* spp., other PAH-degrading organisms, and members of a PAH-degrading community. Consequently, subsequent studies could start by developing qPCR assays for newly identified functional marker-gene candidates — genes encoding for RHDPS1 and the highly transcribed hydratase aldolase (gene CYCPU RS0105800). The respective TtG ratios need to be correlated with the naphthalene-degradation performance of *Cycloclasticus* spp. both in pure culture and within an environmental community, similar to the work presented in Vogel et al., 2023b. Such work should include treatments that grow strain PS-1 on minimal medium as well as *in situ*-like microcosms with seawater from Puget Sound (Gulf of Mexico, US) where strain PS-1 was isolated from (Dyksterhouse et al., 1995). Furthermore, naphthalene concentrations in the ng to µg L^{-1} range could be included to identify

a “threshold” concentration at which the transcription of genes from the $\mathrm{NAP_{pos}}$ category like *rhdPS1*α and CYCPU RS0105800 are induced and investigate the transcription of these PAH-degradation genes under more environmentally relevant conditions. It is also possible that the transcripts (in the form of mRNA) for the genes from the different enzymatic systems are degraded differently within the cell. Theoretically, the mRNA of enzymes encoded by substrate-independently transcribed genes could be degraded faster and, therefore, would need to be continuously produced. Conversely, mRNA of genes from the $\mathrm{NAP_{pos}}$ and $\mathrm{NAP_{neg}}$ categories could be more stable and, therefore available over a longer time frame. The genes from the $\mathrm{NAP_{pos}}$ and $\mathrm{NAP_{neg}}$ categories would, in this case, potentially only be transcribed occasionally and the observed apparent correlation with naphthalene availability could have been coincidental. Confirming the proposed enzymatic systems in *Cycloclasticus pugetii* strain PS-1 with enzymatic assays and proteomic approaches would be a necessary next step.

Is the modelled impact of dosage concentration and frequency representative of real-world responses?

The presented results from simulated *Cycloclasticus* cultures indicated that the system recovery takes longer for repeated low-concentration contamination events and that this effect is amplified when the time between dosages is longer. Given the highly relevant environmental implications, further research is imperative to confirm the influence of dosage concentration and frequency on PAH-degradation dynamics in laboratory-based experiments. Future studies could start by conducting simple growth experiments with a model organism like strain PS-1 and quantify biomass increase and PAH concentration. Passive samplers and sampling of the gas phase should be used not only to determine the total PAH concentration in the treatments but also to measure the dissolved and gaseous PAH fractions. To further increase the understanding of the system ^{13}C-labelled substrate could be provided and stable-isotope probing could be used to trace the uptake of PAH into the cells and thereby confirm that the dissolution of PAH and not the uptake of the PAH into the cell is indeed the rate-limiting step for biodegradation. Such experiments could subsequently be extended to test the biodegradation dynamics of the model organism with hydrocarbon mixtures supplied at different dosages (e.g., below solubility, below the concentration at which a slick is formed, and above such a concentration). Moreover, *in situ*-like microcosm studies could investigate the effect of dosage concentration and frequency of pure PAHs and PAH mixtures on environmental microbial communities.

How specific are RHDs in terms of PAH usage and is there a way to predict the affinity to specific PAHs from *in silico* genome analysis?

When consulting the literature or relevant databases about RHDs, there are often specific PAHs like naphthalene, phenanthrene, biphenyl, or pyrene given as substrates which are

targeted by the respective RHD, leaving the impression that the RHDs would exclusively degrade the given PAH. Consequently, based on available literature on *Cycloclasticus* sp. strain P1 and *Cycloclasticus* sp. strain A5, *rhd2*α and *rhd3*α were chosen as target genes for this study, expecting that the transcription would indicate the phenanthrene- and naphthalene-degradation activities, respectively (Vogel et al., 2023b). Surprisingly, the presented results show no substrate-specific transcription of the target genes for naphthalene or phenanthrene in *Cycloclasticus pugetii* strain PS-1, despite their high genomic similarity to the aforementioned *Cycloclasticus* spp. (>98.3% nucleotide-based pairwise identity between the mentioned strains for both, *rhd2*α and *rhd3*α). When expressed in *Escherichia coli*, the RHD3 genes enabled *E. coli* to degrade 83% of naphthalene, however, 21% of phenanthrene, 27% of fluoranthene, and 25% of biphenyl were also mineralized (W. Wang et al., 2018). This contradicts the exclusive use of RHD3 for naphthalene degradation and rather hints at a strong affinity for naphthalene. W. Wang et al., 2018 did not compare transcription levels of the genes encoding for the RHDs under different substrates, hence, it is unclear if the substrate-independent expression of functional genes can hint at a broader PAH range. Further studies could, therefore, repeat these valuable cloning experiments and express the RHDs of interest from our model organism strain PS-1. Such work could quantify the mineralization rates of multiple low molecular weight PAH substrates for RHDs from the two proposed enzymatic systems expressed in *E. coli*, e.g., RHD3/ RHD5 and RHDPS1/RHD9 for the ”background” and the ”rapid response” systems, respectively. Moreover, TtG ratios of the respective target genes could be quantified for strain PS-1 grown on the same substrates and a no-PAH control. Additionally, TtG ratios of the target genes should be quantified in other *Cycloclasticus* spp. grown on different substrates, such as strain P1. Combining these detailed characterizations of enzyme affinity and gene transcription could close the knowledge gap on how the specificity of RHDs towards PAH substrates is reflected in the transcription of the encoding genes. Such studies could be expanded by including comprehensive phylogenetic comparisons of RHDs in other marine PAH-degrading bacteria and linking the genomic features to the individual characterization of RHDs. Thereby, it might become possible to identify genomic and transcriptomic features of RHDs which predict the degree of PAH specificity and the associated enzymatic system on a global level. In the future, analyzing the metagenomes and -transcriptomes of marine communities might be sufficient to recognize the number of ”background” and ”rapid response” PAH-degrading enzymes which could, in turn, predict if a community can respond fast to PAH contamination or not.

Is there a concentration threshold at which the “rapid response” system is activated and is there a way to predict it e.g., from physico-chemical properties of the PAH?

The results presented in this thesis showed that some PAH-degrading genes were transcribed without available PAH substrate, while other genes were transcribed in response to PAH input. The exact PAH concentration necessary to activate the transcription of genes

encoding for enzymes within the “rapid response” system, however, remains unclear. Such a threshold concentration is likely to be below the maximum solubility concentration of the target PAH since the solubility in seawater represents the maximum dissolved and, thus, bioavailable concentration to which *Cycloclasticus* spp. might have adapted. Furthermore, given that environmentally occurring PAH concentrations are in the ng to µg L^{-1} range, more studies need to investigate how degradation dynamics change at low contamination levels. A first step, for example, would be implementing classical Michael-Menton kinetic experiments to determine the biodegradation kinetics when naphthalene concentrations are between the solubility in seawater and zero with a high sampling frequency at lower concentrations. In addition to following the PAH and biomass concentrations, TtG ratios of genes encoding for target genes from both enzymatic systems should be determined using qPCR. An increase in the TtG ratio of target genes from the “rapid response” system, like *rhdPS1*α, should indicate the threshold concentration that activates the corresponding enzymatic system. In contrast, the TtG ratio of genes encoding for enzymes within the “background” system (e.g., *rhd3*α) should be constantly high and can serve as a qualitative positive control. Such experiments could be expanded to other PAH substrates like phenanthrene, biphenyl, or pyrene and could, consequently, determine if there is a correlation between the threshold concentration at which the ”rapid response” system is activated and physico-chemical properties such as the solubility of a target substrate. Moreover, PAH and hydrocarbon mixtures could be tested and by considering the altered solubility of the target PAH within the mixture validate such a proposed correlation. Ultimately, the determined threshold concentrations for the single compounds could be confirmed by conducting *in situ*-like microcosm studies, and exposing environmental communities first to pure PAHs and later to hydrocarbon mixtures.

Are there differences in transcription of PAH-related genes on a single-cell level and if so, which part of the population is active?

Gene expression can vary significantly from cell to cell in a genetically identical microbial population even under constant growth conditions (Sampaio et al., 2022). Although phenotypic variability in transcription is well documented for different bacteria — particularly for genes involved in stress responses like DNA repair or antibiotic resistance — the underlying dynamics are often unknown and the phenomenon has not been reported in PAH-degrading genes to date (El Meouche & Dunlop, 2018; Uphoff, 2018). Nonetheless, such heterogenic expression could be one reason for the presented TtG ratios being below a value of one (Vogel et al., 2023b), indicating that not every individual cell is expressing the PAH-related target genes — even during the exponential phase of growth and active PAH degradation. Therefore, further studies should investigate, if the heterogeneous expression of PAH-degradation genes does occur in *Cycloclasticus* spp. on a single-cell level. A potential first step could involve the use of stable-isotope probing to identify if there is a part of the bacterial population that incorporates significantly more ^{13}C-labelled PAH. By subsequently cultivating those

different parts of the population, it could be determined whether that effect is reversible. Further, single-cell experiments could investigate real per-cell transcription of PAH-degradation genes either with qPCR assays of individual target genes like *rhd3*α and *rhdPS1*α or by using single-cell RNA sequencing. Since quantifying PAHs in such low-volume reaction wells might prove challenging, again using ^{13}C-labeled substrate could be the solution to pinpoint active PAH-degrading parts of the population. In such experiments, the heterogeneous transcription of genes encoding for enzymes in both proposed systems – the ”background” and the ”rapid response” system – should be investigated.

Can expressed PAH degrading genes be classified as core or accessory genes of *Cycloclasticus* spp. and is there a correlation to genes encoding for enzymes from the “background” and “rapid response” systems?

The proposed ”background” and ”rapid response” enzymatic systems possibly mark *Cycloclasticus pugetii* strain PS-1 as an organism that is well adapted to PAH-contamination but it remains to be confirmed that the resulting response is that of an PAH-degrading specialist. A recent study by Peña-Montenegro et al., 2023 used Core-Accessory (CA) Metatranscriptomes of hydrocarbon degraders to identify the underlying mechanisms of key microbial functions and highlighted differences of specialist-vs-opportunistic responses to hydrocarbon exposure. For that, differentially expressed genes from a metatranscriptome were assessed in the context of species-specific pangenomes, that provide thorough descriptions of both core and accessory genes. Species-specific pangenomes are produced from a collection of closely related genomes, usually by grouping genes according to sequence homology. (Delmont & Eren, 2018). The results from Peña-Montenegro et al., 2023 identified *Marinobacter* as a specialized hydrocarbon degrader with the behavior of an r strategist and copiotroph (Singer et al., 2011) due to their dominant expression of accessory genes (93%) in response to hydrocarbon exposure. In contrast, *Colwellia* was found to exhibit an opportunistic strategy in response to hydrocarbon exposure as the CA-Metatranscriptome demonstrated an acceleration of transcription for both accessory genes (59%) and core genes (41%). It remains, however, unknown if expressed PAH-degradation genes in a *Cycloclasticus* pangenome could be categorized as accessory or core genes and, hence, if *Cycloclasticus* spp. can be confirmed as specialized PAH degraders. Further, it is unclear if genes encoding for the two enzymatic systems could be categorized as either core or accessory genes and if, therefore, the evaluation of CA-Metatranscriptomes could be a way to identify the proposed enzymatic systems. Hence, future studies should (i) create a *Cycloclasticus* pangenome and (ii) assess the transcription of PAH-degrading core and accessory genes in response to different PAHs and under varying substrate concentrations. Moreover, such studies should be (iii) connected to enzymatic assays or proteomic analysis to correlate the CA-transcription to the proposed “rapid response” and “background” systems. Following the line of thought from Peña-Montenegro et al., 2023, however, *Cycloclasticus* is often shown

to be a highly specialized PAH degrader and, therefore, is likely to express accessory genes during PAH degradation. This would suggest that the differentiation between genes that are transcribed substrate independently and those that act in response to PAH input using these *in silico* techniques is questionable.

Is there genetic redundancy in key-functional genes of other PAH-degrading bacteria and how can we differentiate between genes from the two proposed enzymatic systems?

Genetic redundancy was identified to provide genetic flexibility and possibly enables *Cycloclasticus* spp. to rapidly respond to fluctuating PAH loads and, thereby, outcompete other community members. It remains unknown, however, if genetic redundancy and the proposed two enzymatic systems are unique to *Cycloclasticus* or if they are a common strategy in highly adapted PAH- and hydrocarbon-degrading bacteria. Further studies should try to close this knowledge gap since extending our understanding of dynamics in hydrocarbon-degrading communities would promote the ability to predict hydrocarbon degradation. Assessing TtG ratios of PAH-related genes in globally available metagenomes and transcriptomes could be a first step in categorizing new target genes that are PAH-independently expressed and transcribed in response to PAH contamination. The transcriptional behavior of these newly identified target genes should then be tested in response to single PAHs and hydrocarbon mixtures by conducting microcosm studies with environmental communities from representative marine habitats. If genetic redundancy can be identified in other relevant PAH-degrading genera, the question remains if populations within the same genus but collected from chronically polluted and truly pristine environments demonstrate the same degree of functional redundancy and, therefore, adaption to PAH contamination. A global comparison of genomes from PAH-degrading bacteria within the same genus could reveal a correlation between genetic redundancy and the contamination degree of the original environment. In future, *in silico* methods like machine learning could be used to identify patterns between functional redundancy, community members, and PAH degradation. Collectively, the answers to these questions could be used to refine future models, potentially enabling us to predict the fate of PAHs in an environment and even the recovery time of a system - all based on functional genes in the metagenome that indicate the level of hydrocarbon degradation adaptation with the microbial community members.

4. Conclusion

The presented results greatly expanded our understanding of concentration-dependent PAH degradation and gene expression in marine environments. It was demonstrated that PAH concentration is a crucial factor that impacts biodegradation activity from the biochemical pathway level to the overall response of an environmental system. Hence, the often neglected parameters of PAH dosage and frequency of release can strongly influence the fate of contaminants within marine environments. As a proof-of-concept, these effects were demonstrated with a specialized PAH degrader which seemingly has a high degree of genetic flexibility. Two different enzymatic response systems were proposed based on genetic redundancy of PAH-degradation genes that could be categorized based on expression in correspondence to PAH availability. The newly proposed ”rapid response” system likely enables a fast reaction to PAH inputs in a need-based manner. In contrast, the enzymatic ”background” system was active regardless of available PAH substrate and might facilitate degradation of PAHs at trace amounts. The thesis also indicated how molecular tools can, in the future, be applied to monitor PAH-degradation activity by using functional genes, for example as a tool during bioremediation. With *rhdPS1*α and CYCPU RS010580 two promising marker gene candidates were identified to develop such a powerful tool in the future. The presented thesis further highlights how an interdisciplinary combination of numerical modelling and wet-lab-based studies can close knowledge gaps. Using process-based modeling, it was demonstrated that pristine environments are potentially more vulnerable to repeatedly released trace amounts of PAH. The results should be further investigated and could advise against oil exploration and shipping lanes in pristine marine environments like the Arctic.

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Appendix

Published papers

The work presented in this thesis resulted in two published journal articles and one manuscript that was under revision when the thesis was submitted in December 2023 but has been published since. Vogel et al., 2023a was published in *Environmental Science and Pollution Research* on the 4$^{\mathrm{th}}$ of April 2023, while Vogel et al., 2023b was published on the 29$^{\mathrm{th}}$ of June 2023 in *Frontiers in Microbiology*. On the 6$^{\mathrm{th}}$ of September 2023, a third manuscript was submitted to *FEMS Microbiology Ecology* for consideration as research article and was published on the 13$^{\mathrm{th}}$ of April 2024 (Vogel et al., 2024). The respective articles and the corresponding supporting-information documents are presented in this appendix.

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RESEARCH ARTICLE

Dosage concentration and pulsing frequency affect the degradation efficiency in simulated bacterial polycyclic aromatic hydrocarbon-degrading cultures

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Abstract

A major source of anthropogenic polycyclic aromatic hydrocarbon (PAH) inputs into marine environments are diffuse emissions which result in low PAH concentrations in the ocean water, posing a potential threat for the affected ecosystems. However, the remediation of low-dosage PAH contaminations through microbial processes remains largely unknown. Here, we developed a process-based numerical model to simulate batch cultures receiving repeated low-dosage naphthalene pulses compared to the conventionally used one-time high-dosage. Pulsing frequency as well as dosage concentration had a large impact on the degradation efficiency. After 10 days, 99.7%, 97.2%, 86.6%, or 83.5% of the 145 mg L^{-1} naphthalene was degraded when given as a one-time high-dosage or in 2, 5, or 10 repeated low-concentration dosages equally spaced throughout the experiment, respectively. If the simulation was altered, giving the system that received 10 pulses time to recover to 99.7%, pulsing patterns affected the degradation of naphthalene. When pulsing 10 days at once per day, naphthalene accumulated following each pulse and if the degradation was allowed to continue until the recovered state was reached, the incubation time was prolonged to 17 days with a generation time of 3.81 days. If a full recovery was conditional before the next pulse was added, the scenario elongated to 55 days and generation time increased to 14.15 days. This indicates that dissolution kinetics dominate biodegradation kinetics, and the biomass concentration of PAH-degrading bacteria alone is not a sufficient indicator for quantifying active biodegradation. Applying those findings to the environment, a one-time input of a high dosage is potentially degraded faster than repeated low-dosage PAH pollution and repeated low-dosage input could lead to PAH accumulation in vulnerable pristine environments. Further research on the overlooked field of chronic low-dosage PAH contamination is necessary.

Keywords Marine oil pollution · Chronic low-concentration pollution · Biodegradation · Chemical fate modelling · Bacterial growth model · Simulation of batch experiments

Introduction

Approximately 1.3 million tons of hydrocarbons are emitted through anthropogenic and natural sources to marine environments every year (National Research Council, 2003). Most prominently known among anthropogenic sources are accidental oil spills and disasters like the Deepwater Horizon accident in 2010, which generate high public and scientific attention. Consequently, current research mainly focuses on one-time high-hydrocarbon input scenarios, however, these account for only 9% of the total emitted hydrocarbons. Another research focus is often natural oil seeps which are continuously polluted extreme environments at the seafloor, comprising 48% of global hydrocarbon emissions (National Research Council, 2003). In contrast, anthropogenic,

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repeating, diffuse and often low-dosage hydrocarbon emissions account for up to 40% of the total but are underrepresented in research. This is mainly because their location and timing are highly variable and they occur in pulses, e.g., due to ships passing on a shipping route, rain events causing increased run-off or atmospheric deposition from burning organic matter or fuels (National Research Council 2003; Duran and Cravo-Laureau 2016). These factors make them hard to identify, quantify, and investigate in situ; thus, knowledge on the impact of repeated low-dosage hydrocarbon pollution remains scarce.

Microbial hydrocarbon degradation is a crucial process for contaminant removal from (marine) environments, such as the ocean water column (González-Gaya et al. 2019). This is well studied for high-pollution events under both oxic and anoxic conditions in aquatic systems, considering different salinities, and temperatures (Gutierrez et al. 2013; Joye et al. 2016), but is not well studied for repeated low-dosage scenarios due to the difficulties mentioned above. When studying hydrocarbons, it becomes apparent that there is a difference between aliphatic and polyaromatic hydrocarbons (PAHs). While aliphatic, chain-like hydrocarbons are degraded faster, PAHs are known to be more persistent and, due to relatively low hydrophilic properties, tend to concentrate and accumulate in organic material and organisms (Baussant et al. 2001; Landrum et al. 2003). Indeed, some PAH compounds have been listed as "Priority Pollutants" by the US EPA since the 1990s due to their persistence, acute toxicity, and carcinogenicity (Environmental Protection Agency 1993).

PAH degradation by marine bacteria is a highly relevant and well-known process, helping the environment to recover from one-time high-input hydrocarbon pollution events (Joye et al. 2016; Karthikeyan et al. 2020; Bacosa et al. 2022). Observed PAH degradation half-lives range from days to months and strongly depend on the complexity of substrate (single compound vs. mixture of hydrocarbons), temperature, UV light and composition of the microbial community. For example, Liu et al. (2016) found temperature-dependent degradation rate constants between 0.101 and 0.399 d^{-1} with PAH residuals between 34 and 75% after 50 days by performing in situ-like microcosm experiments using seawater contaminated with 200 mg L^{-1} crude oil. In comparison, a pure culture of *Cycloclasticus* spp. degraded up to 99% of 200 mg L^{-1} within 10 days under optimized conditions in a laboratory based study by Cui et al. (2014). Knowledge on the degradation dynamics of repeated low-dosage PAH contamination, however, is scarce since small changes in low PAH concentrations are more difficult to study in the laboratory due to technical limitations and biological variability that masks actual changes in low concentration.

To date, only a few studies have investigated repeated low-dosage hydrocarbon degradation in the water column of the ocean. For example, Bacosa et al. (2021) incubated seawater microcosms with different initial oil concentrations for 50 days and environmentally exposed flow-through incubators were studied with two different concentrations of weathered diesel (Ryther et al. 2021). No further in situ, in situ like, lab-based or simulation-based studies are available so far to predict the fate of PAHs in a chronic pollution scenario. It also remains unclear what role the frequency of input and the time interval between regularly occurring pollution events play in the recovery of the system. This knowledge would be highly relevant for risk assessment and contamination management, given that many low-dosage PAH inputs are recurring pulse-like pollution events. Research on other contaminants like herbicides suggests that frequency and dosage concentration play an important role for the degradation efficiency of contaminants (Baelum et al. 2008; Lancaster et al. 2010). Nonetheless, no data has been published so far on pulsing experiments or field studies when it comes to marine hydrocarbons, or more specifically PAH degradation.

Modelling microbial PAH degradation could help start closing the knowledge gap on repeated marine low-dosage hydrocarbon pollution of the water column. Although field and laboratory-based studies are not replaceable in order to understand environmentally relevant processes, they have limits when it comes to temporal and spatial resolution, as well as number of experiments.

Therefore, conceptual models that represent our current understanding of interacting processes and driving parameters related to microbial PAH degradation in aqueous environments can be used as an alternative, ultimately, translating our experimental knowledge into mathematical equations (Soulas and Lagacherie 2001; Brimo et al. 2016). Numerical simulations can complement microbiological experimental research and help to analyse available data and derive parameter values and degradation kinetics. Based on scenario analysis, study results can be transferred to different conditions, habitats, or environmental scales and the experimental design for follow-up studies can be improved. Additionally, using mathematical model simulations with data from previously conducted experiments can help to understand dynamics in between sampling points and beyond the sampling time. This also allows the sampling design for future experiments to be adapted (if required), e.g., by choosing sampling times and scales according to the most "critical" dynamics as projected by the model. Furthermore, model simulations can be used as a first step to investigate different hypotheses, e.g., on driving parameters, to refine experimental design and generate preliminary data sets. More specifically, utilizing data on microbial degradation in one-time high-dosage scenarios (Wang et al. 2018) can inform simulations of repeated low-dosage microbial degradation. This can ultimately help to anticipate the fate

of unexplored repeated low-dosage oil input within marine environments.

The overarching aim of this study was to simulate and understand recovery times of a batch culture for different PAH input scenarios, by comparing one-time high-dosage to repeated low-dosage inputs. Therefore, i) a numerical model simulating a single-strain batch culture of a known marine PAH-degrader was developed; ii) parameter values were derived by applying our model to available literature data on batch culture studies; and iii) scenarios were run to predict the dynamics of biomass growth and PAH concentrations over the course of simulated one-time high-dosage vs. repeated low-dosage degradation experiments.

Methods

Experimental data

Wang et al. (2018) performed one-time high-input batch culture studies with different PAHs in order to analyse the degradation pathway, on a genetic level, using the isolated marine model organism *Cycloclasticus* sp. strain P1, a strain from a species of well-known key players in marine PAH degradation (Dyksterhouse et al. 1995; Geiselbrecht et al. 1998; Dombrowski et al. 2016). They followed cell density and total PAH residual percent of the initial concentration for ten days (Fig. 1 in Wang et al. (2018)). Their growth experiment is briefly summarized here since its data are the basis for our model*; Cycloclasticus* sp. strain P1 was grown in 100 ml oxic artificial seawater medium at 28 °C on a rotary shaker (150 rpm) with 500 ppm naphthalene as the sole carbon source for 10 days. Naphthalene residuals were measured via HPLC, bacterial growth was monitored with optical density measurements at a wavelength of 600 nm (OD_{600}). Further details can be found in Wang et al. (2018). We used the published growth observations as biomass concentration (after conversion from OD_{600} in cells L^{-1}, Eq. ES1, SI) and naphthalene concentrations in mg L^{-1}, calculated from the naphthalene residual data as input for model calibration (Table S1).

Conceptual model

PAH is assumed to be present in different states, i.e., gaseous, dissolved in the aqueous phase, and crystalline (Fig. 1). In the model, these PAH fractions, as concentrations (mg L^{-1}), were assigned to three of the state variables of the model, C_{gas}, $C_{crystal}$, and C_{diss}, respectively. After being emitted as a pure compound or in a mixture with different PAHs and solvents, evaporation and spreading/dilution of the crude oil compounds would lead to a decrease in the PAH concentration under environmental conditions. Aside from photooxidation at the seawater surface, biodegradation is the main process that removes PAHs from the water column. Therefore, the fourth state variable of the model is assigned to the biomass of the PAH-degrading bacteria, B in cells L^{-1}.

The model is designed to represent batch experimental conditions and is based on model concepts developed by Monod (1949), Volkering et al. (1992), Grimberg et al. (1996), Knightes and Peters (2003), and Brimo et al. (2016). When simulating environmental processes in laboratory batch cultures incubated in the dark, photooxidation as well as spreading and dilution of PAHs can be neglected. The following processes, that link the four state variables, are considered in the initial model structure, that from here on is called reference model (M_{ref}):

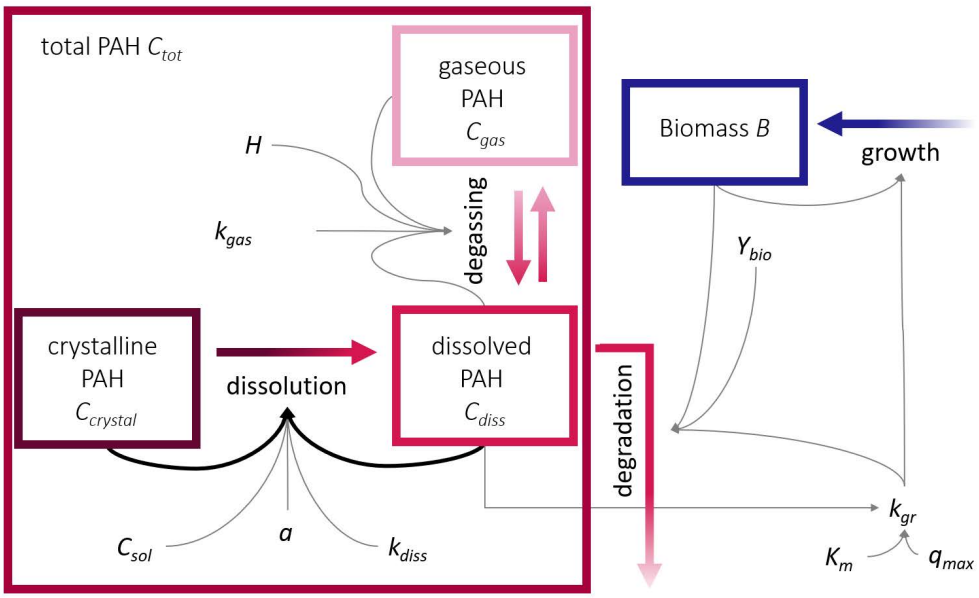

Fig. 1 Overview of the dosage-schemes in different simulated scenarios of the reference model; number of dosages, dosage interval and dosage concentration for scenarios S1 to S6

PAH dissolution: Dissolution of the crystalline PAH ($C_{crystal}$) into dissolved PAH (C_{diss}) is driven by the gradient between the aqueous saturation concentration, i.e., the solubility, of the PAH (C_{sol}) and the dissolved concentration (C_{diss}). The rate of mass transfer across the solid–liquid interface depends, in addition, on the specific surface area of the PAH crystals a (surface area per unit volume, in cm^{-1}) and the mass transfer coefficient for dissolution k_{diss} (in cm h^{-1}) which is, in fact, a function of chemical and hydrodynamic properties. Since a batch culture is a closed system with limited available PAH, $C_{crystal}$ needs to be accounted for as a limited resource for dissolution while C_{sol} is considered as the capacity-term for maximum dissolution of PAH.

PAH degassing: Unitless Henry coefficients of volatile PAHs range from $8.88 \cdot 10^{-6}$ to 3.1110^{-2} (naphthalene to picene, (Sander 2015)) and indicate a distribution process from the aqueous to the gas phase. It remains to be determined if volatilization of PAHs into the headspace is a relevant process in a closed batch system under laboratory conditions. Thus, degassing is considered in the reference model structure and follows the concentration gradient between the dissolved (C_{diss}) and the gaseous PAH (C_{gas}).

PAH degradation: Aerobic PAH-degrading bacteria can oxidize hydrocarbons via complex metabolic pathways, thereby using them as carbon and/or energy source for biomass production and dissimilatory processes which result in CO_2 production (Bouchez et al. 1996; Baboshin and Golovleva 2012; Dombrowski et al. 2016). Considering the lipophilic properties of PAHs and high ionic strength of seawater, solubility is limited, making it a potential rate limiting step in microbial degradation since uptake into the cell and, therefore, bioavailability is strongly linked to the dissolution of compounds. Thus, only the dissolved fraction of PAHs is considered to undergo degradation. PAH degradation is linked to biomass growth and follows Monod kinetics assuming a constant biomass yield per PAH mass (Y_{bio} in $mg_{BIO}\ mg_{PAH}{}^{-1}$).

Bacterial growth: Growth of bacterial biomass B (cells L^{-1}) is assumed to follow Monod kinetics with the Monod constant (or half-saturation constant) K_m (mg L^{-1}) and maximum PAH degradation rate q_{max} in $mg_{PAH}\ mg_{BIO}{}^{-1}\ h^{-1}$. As described above, the biomass yield Y_{bio} is assumed to be constant. Growth is considered to be net growth, i.e., explicit cell death does not play a role during the simulated 10-day period. Production of emulsifying compounds by bacteria is neglected in our model, as is sorption of PAH to biomass.

Model equations

The mass balance model, as outlined above, was formalized into the following system of four differential equations that simulate the dynamics of microbial biomass B (cells L^{-1}) and hydrocarbon concentration in aqueous (C_{diss} in mg L^{-1}), crystalline ($C_{crystal}$, in mg L^{-1}) and gaseous (C_{gas}, in mg L^{-1}) states in a PAH degrading, marine batch culture.

$$\frac{dB}{dt} = k_{gr} \bullet B \tag{1}$$

$$\frac{dC_{diss}}{dt} = k_{aq} \bullet a \bullet C_{crystal} \bullet \left(1 - \frac{C_{diss}}{C_{sol}}\right) - \frac{k_{gr}}{Y_{bio}} \bullet \left(B \bullet 10^{-9}\right) - k_{gas} \bullet \left(C_{diss} \bullet H - C_{gas}\right) \tag{2}$$

$$\frac{dC_{crystal}}{dt} = -k_{diss} \bullet a \bullet C_{crystal} \bullet \left(1 - \frac{C_{diss}}{C_{sol}}\right) \tag{3}$$

$$\frac{dC_{gas}}{dt} = k_{gas} \bullet \left(C_{diss} \bullet H - C_{gas}\right) \tag{4}$$

with the growth rate constant k_{gr} (h^{-1}) as a function of the dissolved PAH concentration C_{diss} following Monod kinetics

$$k_{gr} = q_{max} \bullet \left(\frac{C_{diss}}{K_m + C_{diss}}\right) \tag{5}$$

and the mass transfer coefficient for dissolution k_{diss} (cm h^{-1}), the specific surface area a (cm^{-1}) of the crystalline PAH, the solubility, i.e., saturation concentration of the PAH in the aqueous phase C_{sol} (mg L^{-1}), the biomass yield Y_{bio} ($mg_{BIO}\ mg_{PAH}{}^{-1}$), the mass transfer coefficient for volatilization k_{gas} (h^{-1}), the dimensionless Henry coefficient H (-), the maximum growth rate constant q_{max} (h^{-1}) of the bacteria, and the Monod half-saturation constant K_m (mg L^{-1}). The factor 10^{-9} in the degradation term of C_{diss} (Eq. 2) is a conversion factor for the microbial cellular biomass, i.e., biomass per cell (mg $cell^{-1}$). This system of ordinary differential equations was defined as the reference model structure and was solved numerically with MATLAB R2018a using the in-built solver ode15s.

Model alternatives and sensitivity were analyzed by switching on and off subsets of transfer pathways (processes) between the variables of PAH states and bacterial biomass B, varying parameter dependencies and adopting mathematical descriptions of the assumed kinetics where reasonable.

Model alternatives and sensitivity analysis

A model alternative was developed to investigate the structural uncertainty due to assumptions made about processes and their kinetics. The model alternative maintains the set of four state variables but differs in the assumptions on dissolution kinetics (model alternative M_{lit}).

M_{lit}: In literature, the dissolution term as defined in Eq. 2 and 3 is simplified to follow a first-order rate model driven

by the gradient between maximal soluble PAH (C_{sol}) and the dissolved PAH (C_{diss}) (Perry 1950). Note that this approach does not consider the limited availability of PAH in the closed batch system but has been shown to explain dissolution kinetics in earlier studies, e.g., Volkering et al. (1992), Grimberg et al. (1996). Therefore, the dissolution term in Eqs. 2 and 3 is slightly adjusted in the differential equations for the change of the dissolved PAH and the pure PAH:

$$\frac{dC_{diss}}{dt} = k_{diss} \bullet a \bullet \left(C_{sol} - C_{diss}\right) - \frac{k_{gr}}{Y_{bio}} \bullet \left(B \bullet 10^{-9}\right) - k_{gas} \bullet \left(C_{diss} \bullet H - C_{gas}\right) \quad (6)$$

$$\frac{dC_{crystal}}{dt} = -k_{diss} \bullet a \bullet \left(C_{sol} - C_{diss}\right) \quad (7)$$

Sensitivity: Volatilization of PAHs plays a significant role under environmental conditions when the volume of the atmospheric compartment and, thus, the PAH concentration gradient from the aqueous to the gaseous phase is large (National Research Council 2003). Under laboratory batch conditions, the headspace is small compared to the atmosphere and volatilization might be an instantaneous process. The sensitivity of the model results was tested by varying the value of the mass transfer constant k_{gas} for volatilization in a range of 0.1 to 10,000 h^{-1}.

Derivation of model parameters

Data on total PAH (naphthalene) concentration (C_{tot}) and microbial cells (B) for model calibration and parameter deviation were taken from Wang et al. (2018). Simulating a regular growth experiment where the addition of PAH marks the starting point, the state variables were initially: $C_{crystal,0} = C_{tot,0}$, $C_{diss,0} = 0$, and $C_{gas,0} = 0$. Model alternatives (M_{ref}, M_{lit}) were fitted to the experimental data to derive a set of optimum model parameter values (Table S2-SI). The biomass yield (Y_{bio}), the maximum growth rate constant (q_{max}), and the half saturation (Monod) constant (K_m) as well as the mass transfer constant for degassing of naphthalene into the gas phase (k_{gas}) and the coefficient for dissolution of naphthalene into the aqueous phase (k_{diss}) alongside with the specific surface area (a) were estimated using nonlinear least-squares fitting implemented in the computer software MATLAB (lsqnonlin). Lower (0 for all parameters) and upper boundaries (1 h^{-1} for q_{max}, 1000 for all other parameters) for the parameter values were defined for the fitting procedure to limit the search space. To avoid overemphasizing the biomass data during the fitting procedure due to comparably high cell concentration versus total PAH concentration, biomass and naphthalene concentration data (both measured and simulated) were normalized by the respective maximum measured value.

For model comparison, the Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and the normalized root-mean-square error (NRMSE) were used as indicators for the goodness-of-fit of the models to experimental data. Additionally, AIC_{norm} and BIC_{norm} were calculated using normalization by the respective maximum measured value in order to equally regard errors in all model parameters, regardless of their absolute value. All indicators consider the deviation of the model results from the actual data points. AIC is a relative indicator for the model fit and takes the number of fitted parameters and the size of the underlying dataset into account. It rewards the goodness of fit to the data while punishing model complexity, i.e., a high number of fitted parameters that might lead to overfitting. BIC, similar to AIC, uses the maximum log-likelihood to evaluate and compare different models. In contrast to AIC, however, it punishes complexity even more by giving models with an increased number of model parameters a higher score. NRMSE, AIC, and BIC are smallest for the most appropriate model. In addition to the statistical criteria, a graphical analysis, undertaken by inspecting the visual deviations between model results and measurements, supports the determination of the most appropriate model approach.

Model application for experimental design

The optimized model was used to analyze six setups (model scenarios) for microbial laboratory-based degradation experiments that differ in PAH dosage and pulsing frequency by simulating naphthalene degradation and growth of the model organism *Cycloclasticus* sp. strain P1 (Table 1). A common experimental setup of a one-time high-dosage PAH-degradation experiment, reflecting, e.g., an accidental oil spill, was compared to scenarios where the same total mass of naphthalene was applied at equal intervals over the total experimental time of 10 days, representing e. g. repeated river runoff. For the one-time high-dosage scenario (S1), 500% of

Table 1 Overview of the dosage-schemes in different simulated scenarios with the reference model

Scenario	Number of dosages	Dosage interval [days]	Dosage concentration [mg L^{-1}]	Total simulated incubation time [days]
S1	1	-	144.79	10
S2	2	5	72.40	10
S3	5	2	28.96	10
S4	10	1	14.48	10
S5	10	1	14.48	16.9
S6	10	6.12	14.48	55.1

the calculated naphthalene solubility in the given system was selected (solubility = 28.96 mg L^{-1} with salinity 0.612 mol L^{-1} and Setschenow constant for naphthalene in seawater 0.256 L mol^{-1}; Xie et al. 1997). Correspondingly, for the three repeated low-dosage scenarios subsequently lower concentrations per dosage were chosen. Two times 250% (S2), five times 100% (S3) and ten times 50% (S4) of 28.96 mg L^{-1} were added at equal intervals during the simulated experiments, thereby predicting biomass dynamics and naphthalene concentration in the different states (crystalline, gaseous, dissolved).

Degradation performance and the recovery of the batch culture after a repeated low-dosage contamination was investigated by simulating how long it would take to have the added naphthalene degraded to the same extent as in S1 after 10 days. Therefore, for scenario S5, a simulation of S4 was run with low PAH pulses equally added during the 10 days of the experiment and, afterwards, biomass and PAH dynamics were left unaffected by further inputs. Recovery time was measured while the residual naphthalene slowly faded. For scenario S6, S5 was adjusted as such that time between pulses was elongated, allowing the system to recover before a new pulse was added.

All simulation results were plotted using MATLAB. Figure 1 was created in Microsoft PowerPoint.

Results

Reduced complexity of the reference model

Both model structures, i.e., M_{ref} and its alternative M_{lit}, were fitted to the available experimental data from Wang et al. (2018) to simulate naphthalene degradation in a closed batch system by *Cycloclasticus* sp. strain P1. All indicators for the goodness of fit AIC/AIC_{norm}, BIC/BIC_{norm}, and NRMSE indicated relatively good fits and were in favor of M_{ref} (Table 2). Additionally, plotting the simulated biomass and total naphthalene concentrations of the two models in comparison to the experimental data from Wang et al. (2018) showed that M_{ref} represented the data better than M_{lit} (Fig. 2). This means that the statistical criteria fit the observations if the availability of the crystalline PAH, $C_{crystal}$, is assumed to be the limiting factor for the dissolution kinetics and C_{sol} is the upper boundary, i.e., the capacity-term of the dissolved naphthalene concentration.

Additionally, the model structure of M_{lit} resulted in a discontinuous development of simulated biomass and naphthalene concentrations around day 5 when crystalline naphthalene concentration was almost zero (Fig. 2 and Fig. S1-SI). Since the dissolution term of M_{lit} (Eqs. 6 and 7) is driven by the gradient between maximal soluble naphthalene (C_{sol}) and the dissolved naphthalene (C_{diss}), a conditional assignment was created for the case where $C_{crystal} > k_{diss} \bullet a \bullet \left(C_{sol} - C_{diss}\right)$ was false. This condition leads to the immediate dissolution of the remaining crystalline naphthalene in a final dissolution

Table 2 Fitted parameters and goodness-of-fit indicators (*NRMSE* normalized root-mean-square error, *AIC* Akaike Information Criterion, *BIC* Bayesian Information Criterion, AIC_{norm} normalized AIC, BIC_{norm} normalized BIC) for the simplified reference model $M_{ref,s}$ and the alternative model structure M_{lit}. Specific surface area a and k_{gas} were set to 1 when model complexity of the reference model M_{ref} was reduced to $M_{ref,s}$ n.a. – no literature data available

Parameter	Literature values	$M_{ref,s}$	M_{lit}	Parameter description
q_{max} [h^{-1}]	0.3[a] – 0.636[b,c]	0.281	0.543	Max. growth rate constant[a,b,c]
K_m [mg_{NAP} L^{-1}]	0.291[c] – 0.572[b,c]	1.000	0.499	Monod constant, half-saturation conc.[b,c]
Y_{bio} [mg_{BIO} $mg^{-1}{}_{NAP}$]	0.17[d] – 0.775[c]	0.208	0.276	Biomass yield[b,c,d,e]
k_{diss} [cm h^{-1}]	1.83E-3[f]	2.5E-2	1.86E-1	Mass transfer coefficient for dissolution of PAH into aqu. phase, dependent on PAH particle size and diffusion coefficient[a,f,g]
a [cm^{-1}]	n.a	1	0.775	Specific surface area of PAH
$k_{aq} = k_{diss} \cdot a$ [h^{-1}]	n.a	0.025	0.144	Mass transfer constant for dissolution of PAH into aqueous phase
k_{gas} [h^{-1}]	n.a	1	0.856	Mass transfer constant for degassing of PAH into gas phase
NRMSE [-]		2.725	4.446	Normalized root-mean-square error
AIC [-]		323.7	334.2	Akaike Information Criterion
BIC [-]		323.4	333.9	Bayesian Information Criterion
AIC_{norm} [-]		145.8	156.3	Akaike Information Criterion, normalized by max. values
BIC_{norm} [-]		145.6	156.0	Bayesian Information Criterion, normalized by max. values

[a]Volkering et al. (1992), [b]Knightes et al. (2003), [c]Knightes et al. (2000) [d]Annweiler et al. (2000), [e]Bouchez et al. (1996), [f]Grimberg et al. (1996) this value was estimated for phenanthrene in a non-marine system and is given as a measure for an order of magnitude since no naphthalene specific data were available, [g]Mulder et al. (2001); (Brimo et al. 2016)

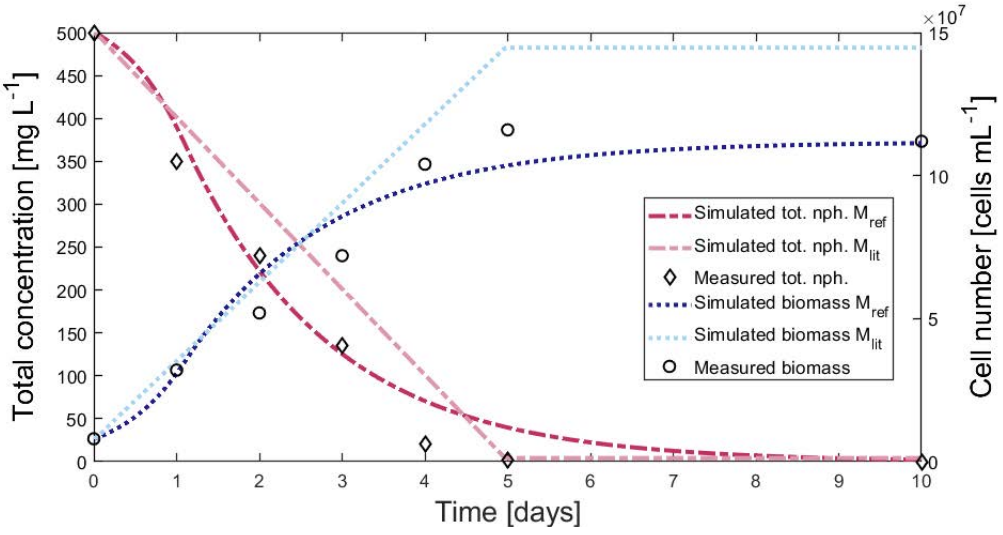

Fig. 2 Comparison of simulated biomass (blue) and naphthalene (pink) concentrations over time by M_{ref} (dark color) and M_{lit} (lighter colour) to experimental results from Wang et al. 2018

step before no more dissolution was possible, since there was no crystalline naphthalene left. This if-condition created an unsteady development of the crystalline and dissolved naphthalene concentrations, which in turn affected the concentrations in gaseous concentration and biomass growth. By treating C_{sol} as a capacity-term (Eqs. 2 and 3) and thereby not requiring a conditional assignment, the model structure of M_{ref} was more robust against discontinuous developments.

In order to avoid over-parameterization, the model structure M_{ref} was tested for the necessity of the processes considered, i.e., for their sensitivity towards process parameters. The specific surface area a of naphthalene—a parameter which is very challenging to estimate in a laboratory experiment and for which no literature data for the given conditions could be found—was arbitrarily set to 1 with the product of a and k_{diss} fitted as a bulk parameter constant (k_{aq} in h^{-1}). Fitting the model with both free parameters vs. keeping one parameter constant at 1 and fitting the other, resulted (and needs to result) in the same product of $k_{diss} \cdot a$ and improved goodness-of-fit indicators due to the reduction in parameters to be fitted and, thus, in model complexity. A sensitivity analysis for k_{gas}, testing values between 0.1 and 10 000 h^{-1}, showed that the model results are insensitive to changes in k_{gas} (Fig. S2-SI and Fig. S3-SI, Table S3-SI). Considering a low k_{gas}, meaning a kinetic inhibition of degassing, is physico-chemically not reasonable and not observed in the laboratory. Thus, k_{gas} was set to 1 h^{-1} and thereby excluded from further simulations which resulted in the following simplified model structure $M_{ref,s}$ of the reference model.

$$\frac{dB}{dt} = k_{gr} \bullet B \tag{8}$$

$$\frac{dC_{diss}}{dt} = k_{aq} \bullet C_{crystal} \bullet \left(1 - \frac{C_{diss}}{C_{sol}}\right) - \frac{k_{gr}}{Y_{bio}} \bullet \left(B \bullet 10^{-9}\right) - \left(C_{diss} \bullet H - C_{gas}\right) \tag{9}$$

$$\frac{dC_{crystal}}{dt} = -k_{aq} \bullet C_{crystal} \bullet \left(1 - \frac{C_{diss}}{C_{sol}}\right) \tag{10}$$

$$\frac{dC_{gas}}{dt} = C_{diss} \bullet H - C_{gas} \tag{11}$$

$$k_{gr} = q_{max} \bullet \left(\frac{C_{diss}}{K_m + C_{diss}}\right) \tag{12}$$

with the bulk dissolution rate constant k_{aq} in h^{-1} (which is, internally, a function of the specific PAH surface area a in cm^{-1} and the dissolution coefficient k_{diss} in cm h^{-1}, $k_{aq} = k_{diss} \bullet a$).

Derived model parameters

The resulting set of fitted model parameters for both model structures, the simplified reference model $M_{ref,s}$ and M_{lit}, was in good agreement with and in the same order of magnitude (except k_{diss}) as the available literature data, i.e., for q_{max}, K_m, Y_{bio} (Table 2). The biomass independent maximum growth rate constant per biomass yield (q_{max}/Y_{bio}) resulted in 1.35 mg NAP ($M_{ref,s}$) to 1.97 mg NAP (M_{lit}) per mg microbial biomass and hour (literature: 0.39–3.74 mg_{PAH} mg_{BIO}^{-1} h^{-1}). The dissolution rate coefficient k_{diss} was overestimated by one ($M_{ref,s}$) to two (M_{lit}) orders of magnitude in comparison to the only available literature value, which was, however, determined for phenanthrene in freshwater medium (Grimberg et al. 1996). Literature values describe kinetics observed in PAH degradation studies in soils, sediment, or freshwater. This might explain the deviation, especially for dissolution kinetics for a marine water column system with high initial naphthalene concentration.

Specifically, degradation studies by *Cycloclasticus* sp. strain P1, the model organism investigated in the underlying studies for this model application, are yet to be conducted.

One-time high-dosage vs. repeated low-dosage

Using the model to compare naphthalene degradation and biomass growth of *Cycloclasticus* sp. strain P1 in one-time high-dosage and repeated low-dosage batch cultures showed that a high PAH contamination scenario is potentially degraded faster than regularly pulsed low-concentration emissions due to the increased dissolution kinetics that dominate substrate availability. This is reflected in four different contamination scenarios (Fig. 3a–d). Simulating an incubation time of 10 days, highest biomass concentration ($3.80 \cdot 10^7$ cells mL^{-1}) and lowest naphthalene residuals (99.7% degradation) were observed in, S1 where the total amount of naphthalene (144.8 mg L^{-1} = 500% maximal solubility) was added in a one-time high-dosage at the beginning of the experiment, representing a high-input event like an oil spill (Table 3). In contrast, the lowest final degradation (83.5%) was predicted after 10 days in S4, where the same total amount of naphthalene was pulsed daily (14.48 mg L^{-1} each = 50% of maximal solubility) to represent a chronic, diffuse naphthalene source such as repeating emissions. A similar final magnitude of biomass was reached at the end of all scenario simulations, while the concentration of PAH residuals varied remarkably between 0.4 mg L^{-1} (0.3% of the total added PAH amount in scenario S1) and 24 mg L^{-1} (16.5% of the total PAH amount in scenario S4; Table 3). Growth patterns and degradation dynamics were highly influenced when the total substrate mass was pulsed in partial quantities rather than being available at once in the beginning (Fig. 3a–d). The higher the frequency of the pulses the more naphthalene accumulated since microbial

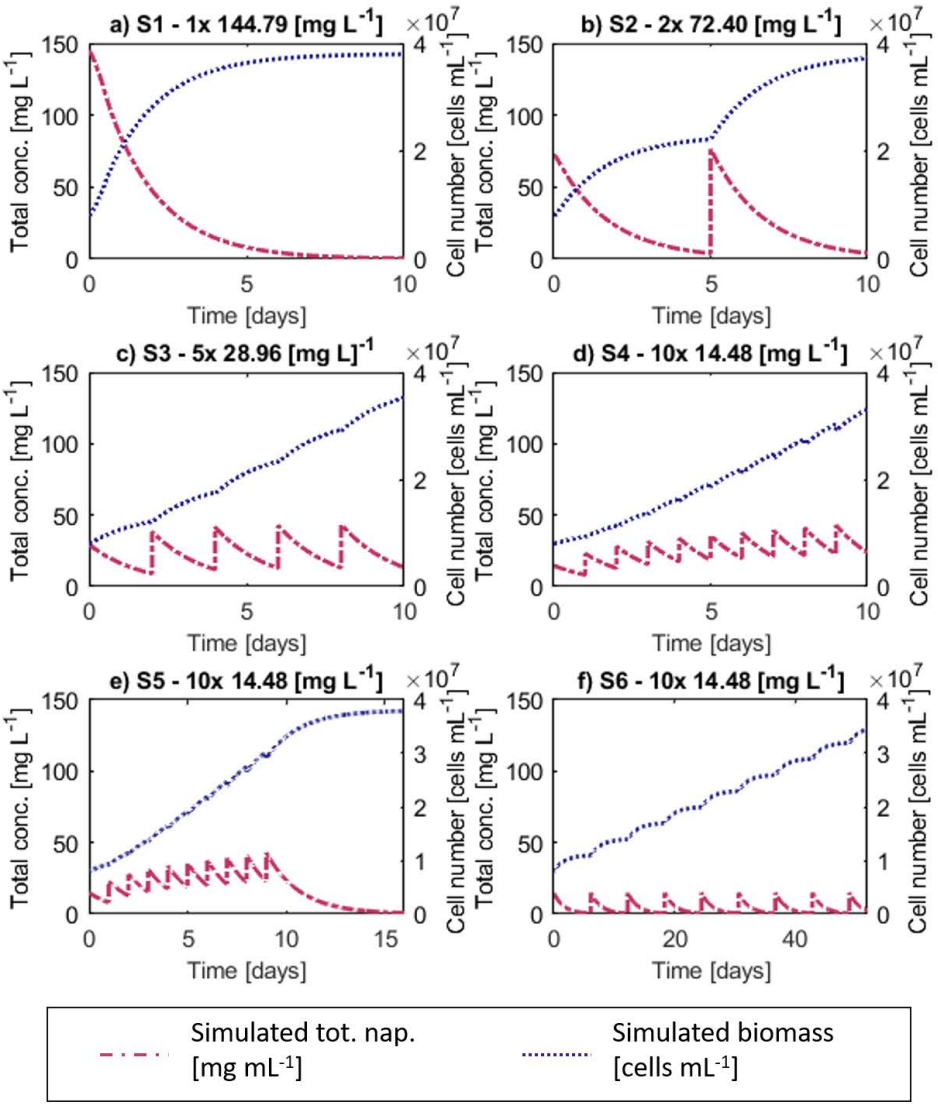

Fig. 3 Total naphthalene (pink, dot dash) and biomass concentrations (blue, dotted) over time for the investigated scenarios. Note that x-axis for fading and elongating scenarios (S5 and S6) differ, indicating longer running times. Simulated biomass and naphthalene concentration over time for different input) scenarios. S1: High input scenario one-time dosage of 144.79 mg L^{-1} (500% max. solubility concentration). S2: Two medium dosages of 72.40 mg L^{-1} (250% max solubility concentration). S3: Five dosages of 28.96 mg L^{-1} (100% max. solubility concentration). S4: Ten dosages of 14.48 mg L^{-1} L (50% max. solubility concentration). S5: After ten dosages of 14.48 mg L^{-1} with a daily frequency, PAH degradation takes about 16 days to reach the same concentration as in S1 (0.4 mg L^{-1}). S6: Low-concentration pulse (14.48 mg L^{-1}) is degraded to the same PAH concentration as at day 10 in S1 (0.4 mg $L.^{-1}$) before the next pulse is given to the system (incubation time for degradation of ten pulses 55 days)

Table 3 Comparison of simulated final naphthalene (nap) and biomass concentrations, overall nap mass loss (degradation) at the end of the simulation and incubation times for the investigated scenarios. Generation times were calculated to highlight retardation of growth despite the similar final biomass concentrations in the different scenarios

Scenario	Incubation time [days]	Final biomass concentration [cells mL^{-1}]	Final nap. concentration [mg L^{-1}]	Nap. degradation [%]	Generation time [days]
S1: 1×144.8 [mg L^{-1}]	10	$3.80 \cdot 10^7$	0.42	99.7	0.65
S2: 2×72.40 [mg L^{-1}]	10	$3.72 \cdot 10^7$	4.03	97.2	1.31
S3: 5×28.96 [mg L^{-1}]	10	$3.53 \cdot 10^7$	13.4	86.6	3.17
S4: 10×14.48 [mg L^{-1}]	10	$3.31 \cdot 10^7$	23.8	83.5	3.81
S5: 10×14.48 [mg L^{-1}]	16.9	$3.80 \cdot 10^7$	0.42	99.7	3.81
S6: 10×14.48 [mg L^{-1}]	55.1	$3.50 \cdot 10^7$	0.40	99.7	14.15

growth and degradation kinetics in between two pulses could not counterbalance the addition of naphthalene into the batch system. Thus, recovery of the system for chronic contamination was investigated with additional model scenarios.

Higher recovery time for less-frequent pulsing

The recovery of the system was investigated by adapting the repeated low-dosage PAH contamination scenario, S4. The amount of naphthalene per dosage (14.48 mg L^{-1}) remained constant, but the simulations were given more time in order to reach the same extent of naphthalene degradation as observed in the one-time high-dosage scenario S1 (99.7%). In S5, where the pulsing frequency was not altered, but the system was given more time to recover after the last pulse, an additional recovery time of 6.85 days was predicted (Table 3). Moreover, when waiting for the system to fully recover each time before adding the next pulse in S6, an even greater elongation of the experimental time was predicted. The estimated time between each pulse was extended to 6.12 days, leading to a prolongation of the total simulation with ten pulses of 45.13 days in comparison to the initial 10 days of simulation. This indicates that not only naphthalene concentration, but also pulsing frequency highly influences the system recovery.

Discussion

Comparison of high one-time and low repeated dosages

One-time highly concentrated PAH emissions are degraded more quickly than repeatedly introduced low-concentration emissions due to the dissolution kinetics that dominate the substrate availability. The gradient between pure and dissolved PAH was the main driving force for the degradation kinetics in a closed batch system. The greater the concentration difference between the pure phase reservoir and the dissolved PAH, the faster the dissolution kinetics (according to Fick's first law). If $C_{crystal}$ is low (at low-contamination events or at the end of a high-level contamination event) and degradation of the dissolved PAH concentration is limited by the availability of PAH in solution, the fast degradation kinetics lead to a continuously low concentration in the dissolved, bioavailable PAH pool (C_{diss}). This small gradient between $C_{crystal}$ and C_{diss} caused dissolution and the emptying of the PAH reservoir to slow down. As a consequence, this led again to a smaller dissolved, and thereby bioavailable substrate fraction.

A low dissolved substrate concentration results in a retarded increase in biomass, since growth of the bacteria is directly dependent on the bioavailable substrate concentration (Volkering et al. 1993).

This means, that biomass growth alone is not a conclusive measure for PAH degradation in batch experiments. All the simulated scenarios, S1 to S4, had the same order of magnitude of biomass concentration at the end of the simulations. Nevertheless, 99.7% of naphthalene was degraded in S1 and the simulated bacteria, with a generation time of 0.65 days, clearly reached stationary phase in the end. In contrast, only 83.5% of the added naphthalene was degraded in S4, accumulating over time, and the simulated batch culture grew linearly, with a generation time of 3.81 days, not reaching stationary phase by the end of the simulation. This effect was described 30 years ago by Volkering et al. (1992), who found that the growth rate of PAH-degrading microorganisms and the solubility of the substrate are correlated. Their conclusion was that bioavailability is constrained due to, e.g., low solubility of the substrate which restricts microbial growth and leads to a linear increase in biomass, rather than an exponential one. In our study, the slowed growth kinetics, reflected by an increased generation time in the less contaminated scenarios, lead to retarded degradation and thereby an accumulation and higher naphthalene residuals over time. In their review, Johnsen and Karlson (2007) describe how diffuse, low-concentration contamination of PAHs can become enriched in

pristine environments like soils despite the presence of PAH-degrading organisms, due to limited bioavailability of the substrate, in this case caused by sorption. Indeed, breaking the established gradient of a system by mixing is a well-known measure for increasing biodegradation. For example, the constant shaking of bacterial cultures in laboratory experiments or mixing contaminated soil with air in remediation scenarios are processes that enhance the bioavailability of the electron donor or acceptor (PAH and/or oxygen), promoting biodegradation (Sales da Silva et al. 2020). This highlights the importance of both, (1) studying pollutant degradation over longer time spans, even under controlled batch conditions, and (2) measuring hydrocarbon concentration rather than using abundance of PAH-degrading organisms as an indicator for degradation kinetics. So far, this has been discussed but rarely applied in microbial field studies, due to the challenges connected to *in-situ* hydrocarbon degradation experiments (Gontikaki et al. 2018; Liang et al. 2019). However, as shown with the simulation studies here, only observing biomass abundance could lead to an overestimation of naphthalene degradation in low-contamination scenarios.

Depending on the pulsing frequency, low-concentration hydrocarbon pulses could lead to a long-term accumulation of the pollutant rather than nearly complete degradation as described for several pristine areas like alpine lakes or mountain areas (Carrera et al. 2001; Vilanova et al. 2001).

System recovery takes longer for repeated low-concentration contamination events. Lower pulsed PAH concentration lead to less dissolved substrate that is available for PAH-degrading bacteria. This, in turn, prevents PAH-degrading bacteria from growing to high densities during the short times which is a self-enforcing process, and ultimately leads to a retardation of the system's recovery. This effect is amplified when increasing the time between dosages (comparing, e.g., the generation times and incubation times of S5 and S6, Fig. 3e and f, Table 3). The implication is that for a pristine marine system, less frequent low-input contamination takes longer to fully recover from or might even lead to pollutant accumulation in the long-run because the PAH-degrading community is not well established due to low growth rates, inhibited by low substrate availability (Carrera et al. 2001; Vilanova et al. 2001). Given that most anthropogenic PAH contaminations in marine environments occur at low concentration and on a relatively continuous, repeated basis, the environmental relevance for PAH degradation dynamics is significant.

Limitations of the model

Since the model itself as well as the underlying degradation experiment are simplifications of the environmental conditions and processes, the chosen model structure was based on a set of boundary conditions and, therefore, comes with limitations. Model outcomes in this study represent homogeneous, laboratory batch experiments. Since, in this study, the general degradation dynamics and growth patterns were of importance, not the absolute concentration values, only the dissolved fraction of the PAH is considered to be bioavailable without restriction of the general validity. Nevertheless, under environmental conditions, additional processes like sorption to solid particles, to dissolved organic matter or to biomass like dead cell walls or EPS in bioaggregates might play a role and could even increase the effects we observed, since bioavailability of the PAH would be even lower (Volkering et al. 1992). Microbial degradation of PAHs might be adversely affected by the PAH concentration itself, e.g., by a toxicity effect at high PAH concentration or by a concentration threshold below which bacteria stop degrading the PAHs, though no such effects have been reported for naphthalene in the literature so far. In addition, dissolution can be actively enhanced by bacteria, e.g., due to the excretion of biosurfactants and bioemulsifiers (Bozzi et al. 1996; Grimberg et al. 1996; Mulder et al. 1998; Qin et al. 2007). However, Gutierrez et al. (2018) found that *Cycloclasticus* spp., isolated near the highly damaged Deepwater Horizon oil rig in 2010, were not capable of producing such biosurfactants, so this was neglected for the PAH biodegradation by *Cycloclasticus* spp. in our model. When extending the model to include several bacterial strains or a complex microbial community, the effect of biosurfactants should be considered since the same study suggested that *Cycloclasticus* spp. jointly with other oil-degrading bacteria were able to benefit from the emulsifying properties of such biosurfactants.

Different physico-chemical properties of aromatic pollutants other than naphthalene could change the observed degradation behavior. Given that naphthalene is a relatively hydrophilic PAH, resulting in a comparably high solubility (even in seawater), the one-time high-dosage scenario that showed a fast degradation process might also be retarded for substances with slower dissolution kinetics (Volkering et al. 1992). Nevertheless, the relative pattern between the input scenarios theoretically should not change when transferring the results for naphthalene to other organic compounds if the conditions are kept the same. Mulder et al. (2001) simulated the biodegradation of four PAHs with different mass transfer parameters such as water solubility and sorption coefficients and found similar patterns of degradation, which were governed by the physico-chemical properties of the PAHs. They concluded that extrapolating kinetic information for different PAHs can be done under mass-transfer limited conditions because the mass-transfer processes are then dominated by the physico-chemical properties of the pollutants.

The elongated degradation times and accumulation of PAHs that we observed for repeated low-concentrated hydrocarbon input is especially relevant for pristine environments. These rely on the establishment of a PAH-degrading community for decontamination, which in turn needs PAH to grow and sustain itself. Studies suggest, that in chronically

polluted areas where low levels of PAHs constantly exist, the native bacterial community adapts and enriches PAH-degrading organisms, probably from the "rare biosphere" and is therefore able to respond faster to PAH contamination (King et al. 2015; Kleindienst et al. 2016; Cerro-Gálvez et al. 2021). Thus, the observed effects from our study are potentially not as severe for such chronically polluted environments and further simulations should investigate this aspect.

In marine environments, contamination is rarely caused by a single PAH, but a complex hydrocarbon mixture like crude oil or diesel, containing aliphatic, branched, cyclic, and polyaromatic hydrocarbons which interact and influence the mass transfer between the compartments of the system (Ghoshal and Luthy 1998; Guha et al. 1999; Ribicic et al. 2018). On top of this, not a single strain, but a complex community evolves to degrade the compounds, all influenced by environmental factors like salinity, UV light, pressure, shoreline energy, mineral particles, nutrients, and temperature (Bargiela et al. 2015; Ward et al. 2018; Piccardi et al. 2019; Bacosa et al. 2022). To begin to understand the effect of (re-occurring) low-concentration oil contamination and expand the model to account for all these variables, further research both in the field and in the laboratory is necessary to tease apart the tangled interlinkages between them.

Conclusion

The model outcomes in this study indicate that it takes longer for a system to recover from less frequent and repeated low-concentration hydrocarbon contamination events. Therefore, events like contamination due to shipping might lead to pollutant accumulation over time. These findings are even more relevant, considering that low-concentration hydrocarbon contamination events occur more often than one-time high-dosage contamination events like accidental oil spills. Additionally, such diffuse events are, in general, a challenge to monitor and, thus, are overlooked. Furthermore, pristine environments are more likely to suffer from low-concentration hydrocarbon contamination since the system is not adapted to PAH-input and, therefore, PAH-degrading bacteria are potentially less abundant and active. This highlights the vulnerability of regions like the Arctic Ocean towards even sporadic low-dosage hydrocarbon contamination. Ultimately, awareness needs to be raised and efforts need to be increased to protect such vulnerable areas against diffuse chronic contamination.

Further studies are needed to investigate contaminated marine systems with high temporal and spatial resolution. More complex experiments and models with PAH mixes or crude oil as well as field studies in marine habitats are required to discern the bioavailability and degradation efficiency of low-concentrated hydrocarbon contamination. This is of particular importance considering the different parameters that influence the bioavailability of hydrocarbons such as the solubility of PAHs in the presence of co-solvents, the salt concentration, and the presence of sediment particles or biosurfactants. Field campaigns that sample habitats influenced by regular low-concentration hydrocarbon contamination—e.g., shipping routes—over timescales from days to months could determine disruptions and in situ recovery times for these ecosystems that might also adapt to the pollutant pressure. Collectively, these data would be needed to confirm the trends we simulated and to increase the model complexity, i.e., to test our advances in system understanding. Our study is the foundation that can be used to better constrain the interactions of coupled pollutant degradation and microbial growth processes and may have important implications for future management and risk assessment of diffuse oil contamination in the marine environment.

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Data availability The MATLAB code used to generate the simulations in this study is available from the corresponding author on reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Supplementary Information

Dosage concentration and pulsing frequency affect the degradation efficiency in simulated bacterial polycyclic aromatic hydrocarbon-degrading cultures

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$$\frac{cells}{mL} = OD * 8 * 10^8 \quad \text{(ES1)}$$

Conversing OD values into cell numbers can be done with a linear equation, when more cells correspond to higher turbidity, which is the case for many cultures growing on aqueous medium during exponential phase (Madigan et al., 2010).This conversion is depending on factors like cell size and shape, the used growth medium and substrate and therefore should be calculated for every investigated system by conducting a calibration experiment. But since those data are not available for the experimental data from Wang et al. (2018), we resorted to using a linear relationship from a lab protocol, which is commonly used for *E.coli* to convert cell densities obtained by measuring the optical density photo-spectrometrically in our lab (Equ. ES2). This general conversion was applied, assuming the resulting cell numbers are at least in the right order of magnitude and nonetheless illustrate the trend the biomass concentration follows over time. Comparing the resulting cell numbers (Table S1) to our own growth experiments with *Cycloclasticus pugetii* PS-1, a very close relative of *Cycloclasticus* sp. P1 (100% nucleotide pairwise identity of the 16S rRNA genes, 88% nucleotide pairwise identity of the available genomes due to NCBI data base August 2022), the calculated cell numbers were reasonable (Vogel et al., in prep.).

Equation to estimate maximal solubility concentration in seawater c_{sol}^{sea} (mg L^{-1}) based on the maximal solubility concentration in freshwater c_{sol}^{fresh} (mg L^{-1}), the salt concentration in the seawater C_{sal} (mol L^{-1}), and the Setschenowv constant k_s (L mol^{-1}) (Gold et al., 1989; Xie et al., 1997).

$$c_{sol}^{sea} = c_{sol}^{fresh} * e^{\left(\frac{(-k_S * C_{sal})}{2.3}\right)} \quad \text{(ES2)}$$

Dosage concentration and pulsing frequency affect the degradation efficiency in simulated bacterial polycyclic aromatic hydrocarbon-degrading cultures - SI Figures

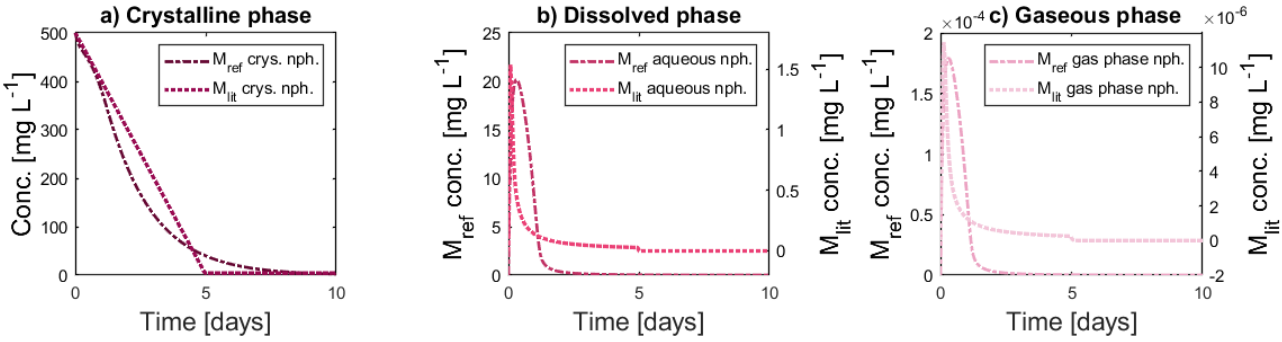

Fig. S1 Comparison between M_{lit} and M_{ref} of the naphthalene distribution between the crystalline, dissolved and gaseous phases in the simulated system

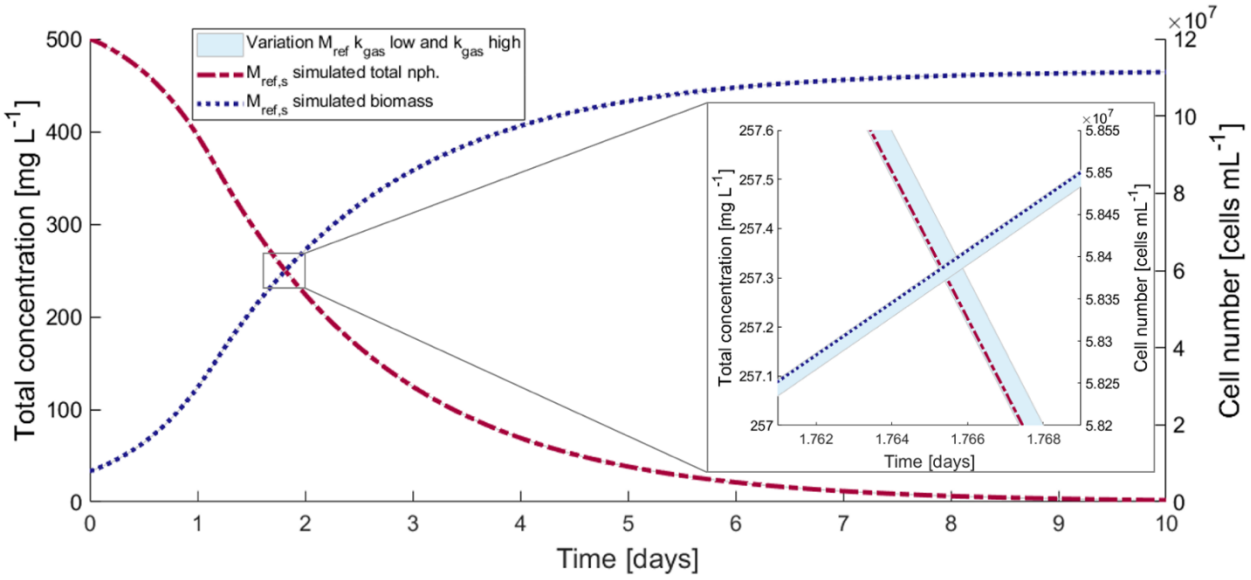

Fig. S2 Variation of $M_{ref,s}$ simulated total naphthalene and biomass concentration over time with k_{gas} low = 0.1 [1/h] and k_{gas} high = 10 000 [1/h]. Neither increasing nor decreasing k_{gas} over several magnitudes does affect the results severely

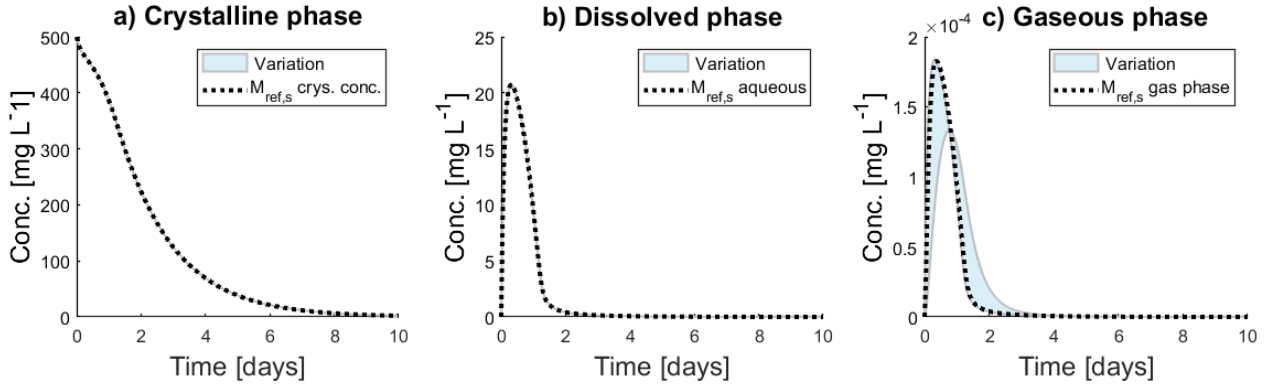

Fig. S3 Variation of M_{ref} simulated total naphthalene concentration in crystalline, aqueous and gaseous phases over time with k_{gas} low = 0.1 [1/h] and k_{gas} high = 10 000 [1/h]. Increasing k_{gas} further over several magnitudes does not affect the results visibly. Decreasing it below 1 does start to impact the distribution in the gaseous phase

Dosage concentration and pulsing frequency affect the degradation efficiency in simulated bacterial polycyclic aromatic hydrocarbon-degrading cultures - SI Tables

Table S1 Experimental results used for parameter fitting (Wang et al., 2018). Naphthalene residuals were converted to concentrations using the reported initial concentration of 500 [mg/L]. Cell densities were used to roughly estimate cell numbers in [cells mL^{-1}] using equation ES1

Days	0	1	2	3	4	5	10
Nph. residuales [%]	0	30	52	73	96	98	100
Cal. nph. conc. [mg L^{-1}][a]	500	350	240	135	20	2	0
Cell density (OD_{600})	0.1	0.4	0.65	0.9	1.3	1.45	1.4
Cell nmb. [cells mL^{-1}][a,b]	$8.0*10^7$	$3.2*10^8$	$5.2*10^8$	$7.2*10^8$	$1.04*10^9$	$1.16*10^9$	$1.12*10^9$

[a]data used for parameter fitting, [b]estimated cell numbers calculated using ES1

Table S2 Input model parameters from literature and initial values for the fitted parameters

Parameter	Value	Reference
Setschenow constant k_s for nph. in seawater [L mol^{-1}]	0.256	(Xie et al., 1997)
Max. saturation concentration c_{sol}^{fresh} in freshwater [mg_{NAP} L^{-1}]	3.10E+1	(Yalkowsky et al., 1983)
Salt concentration C_{sal} of seawater [mol L^{-1}]	0.612	(Gold et al., 1989; Xie et al., 1997)[a]
Max. saturation concentration C_{sol} of nph. (solubility in seawater) [mg_{NAP} L^{-1}]	28.96	(Gold et al., 1989; Xie et al., 1997)[b]
Henry constant H for equilibrium of nph. between water and gas phase [-]	8.88E-06	(Ma et al., 2010; Sander, 2015)
Mass of one bacterium [mg $cell^{-1}$]	1.00E-09	(Davis et al., 1973)
Initial conditions for parameter fitting		
Max. growth rate constant q_{max} [mg_{NAP} mg_{BIO}^{-1} h^{-1}]	0.346	(Knightes et al., 2003)
Monod half-saturation constant K_m [mg_{NAP} L^{-1}]	0.572	(Knightes et al., 2003)
Biomass yield Y_{bio} [mg_{BIO} mg_{NAP}^{-1}]	0.35	(Bouchez et al., 1996; Knightes et al., 2003)
Mass transfer coefficient for dissolution into aqueous phase k_{diss} [cm h^{-1}]	0.25	x
Specific surface area of nph. a in [cm^{-1}]	1	x
Particle independent mass transfer into aqueous phase $k_{aq} = k_{diss} \cdot a$ in [h^{-1}]	0.25	x
Mass transfer constant for degassing k_{gas} in [h^{-1}]	1	x

[a]assuming only NaCl contributes to the molarity of 3.5% salinity, [b]calculated with ES2

Table S3 Model parameter and errors of investigated model structures. Main model is $M_{ref,s}$, highlighted in light blue. Parameter values labeled with * were fixed to 1 for the respective fit in order to reduce complexity of the model structure. n.a. – no literature data available

Parameter	Literature	M_{lit}	$M_{ref,s}$	$M_{ref,2}$	$M_{ref,3}$	M_{ref}
q_{max} [mg_{NAP} mg_{Bio}^{-1} h^{-1}]	0.346 – 0.636	0.543	0.281	0.286	0.277	0.290
K_m [mg_{NAP} L^{-1}]	0.291 – 0.572	0.499	1.000	0.950	0.996	0.919
Y_{bio} [mg_{BIO} mg_{NAP}^{-1}]	0.2 – 1.3	0.276	0.208	0.208	0.208	0.208
k_{diss} [cm h^{-1}]	0.00183	0.186	0.025	0.077	0.025	0.069
a [cm^{-1}]	n.a.	0.775	1*	0.318	1*	0.347
$k_{aq} = k_{diss} \cdot a$ [h^{-1}]	n.a.	0.144	0.025	0.024	0.025	0.024
k_{gas} [h^{-1}]	n.a.	0.856	1*	1*	0.540	989.3
NRMSE [-]		4.446	2.725	2.721	2.728	2.720
AIC [-]		334.2	323.7	325.7	325.6	327.8
BIC [-]		333.9	323.4	325.5	325.3	327.5
AIC_{norm} [-]		156.3	145.8	147.9	147.8	150.0
BIC_{norm} [-]		156.0	145.6	147.6	147.5	149.6

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Substrate-independent expression of key functional genes in *Cycloclasticus pugetii* strain PS-1 limits their use as markers for PAH biodegradation

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Microbial degradation of petroleum hydrocarbons is a crucial process for the clean-up of oil-contaminated environments. *Cycloclasticus* spp. are well-known polycyclic aromatic hydrocarbon (PAH) degraders that possess PAH-degradation marker genes including *rhd3α*, *rhd2α*, and *pahE*. However, it remains unknown if the expression of these genes can serve as an indicator for active PAH degradation. Here, we determined transcript-to-gene (TtG) ratios with (reverse transcription) qPCR in cultures of *Cycloclasticus pugetii* strain PS-1 grown with naphthalene, phenanthrene, a mixture of these PAHs, or alternate substrates (i.e., no PAHs). Mean TtG ratios of 1.99×10^{-2}, 1.80×10^{-3}, and 3.20×10^{-3} for *rhd3α*, *rhd2α*, and *pahE*, respectively, were measured in the presence or absence of PAHs. The TtG values suggested that marker-gene expression is independent of PAH degradation. Measurement of TtG ratios in Arctic seawater microcosms amended with water-accommodated crude oil fractions, and incubated under *in situ* temperature conditions (i.e., 1.5°C), only detected *Cycloclasticus* spp. *rhd2α* genes and transcripts (mean TtG ratio of 4.15×10^{-1}). The other marker genes—*rhd3α* and *pahE*—were not detected, suggesting that not all *Cycloclasticus* spp. carry these genes and a broader yet-to-be-identified repertoire of PAH-degradation genes exists. The results indicate that the expression of PAH marker genes may not correlate with PAH-degradation activity, and transcription data should be interpreted cautiously.

KEYWORDS

PAH-degrading bacteria, process-specific marker genes, transcript-to-gene ratio, biodegradation, marine environment, aromatic ring-hydroxylating dioxygenases

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the ocean, toxic for organisms, accumulate in biomass, and many are considered human carcinogens (Environmental Protection Agency, 1993; Landrum et al., 2003; Nikolaou et al., 2009; Stading et al., 2021; Zhang X. et al., 2021). Microbial biodegradation of PAHs is a key mitigation process (Genovese et al., 2014; Overholt et al., 2016; Gutierrez, 2019a). Since the 1970s, microbial degradation of crude oil components, like PAHs, is well known and bacteria, such as *Cycloclasticus* spp., were identified as relying on these toxic compounds to satisfy their energy and carbon demands (Dyksterhouse et al., 1995; Yakimov et al., 2007; Cui et al., 2019).

Cycloclasticus spp. are some of the best studied aerobic PAH degraders that utilize various PAHs, including naphthalene, phenanthrene, and pyrene, as sources of carbon and energy. They can also make use of certain fatty or amino acids (Geiselbrecht et al., 1998; Staley, 2010), are considered ubiquitous in the marine environment (Lozada et al., 2008; Cui et al., 2014; Rizzo et al., 2019), and recognized as early colonizers of the plastisphere due to their potential plastic degradation capabilities (Popovic et al., 2017; Denaro et al., 2020; Yakimov et al., 2022). Several studies reported correlations between *Cycloclasticus* spp. abundances and the presence of hydrocarbons, e.g., during oil spills (Gutierrez et al., 2013; Rivers et al., 2013; Kleindienst et al., 2016; Zhou et al., 2022). For example, Dong et al. (2015) reported relative abundances of *Cycloclasticus* sequences of 0.2 and 64.5% in pristine versus contaminated Arctic sediments, highlighting their key role in degrading petroleum hydrocarbons, particularly PAHs (Yakimov et al., 2007; Dombrowski et al., 2016; Sieradzki et al., 2021). However, the *in situ* rates of PAH degradation and the environmental factors influencing these rates remain largely unknown.

Quantifying PAH-degradation rates through canonical methods are challenging to conduct *in situ*. These methods include time-series hydrocarbon quantification via liquid chromatography or gas chromatography coupled to mass spectrometry or flame ionization detector (Wilkes, 2010) and standardized routine methods that can be used to calculate *in situ* PAH-degradation rates are largely lacking. Therefore, PAH-degradation rates are quantified in the laboratory and often determined as mean values over longer incubation times (Bacosa et al., 2021; Nolvak et al., 2021), which leads to a knowledge gap regarding predictive understanding of the environmental fate of PAHs. Thus, alternative methods are required to improve monitoring of microbial PAH-degradation activities in the environment.

The ratio of mRNA to DNA of functional genes—i.e., the so-called transcript-to-gene (TtG) ratio—can serve as a cultivation-independent measurement that potentially correlates with per-cell degradation rates and could, thus, provide an *in situ* method for PAH-degradation rate estimation (Baelum et al., 2008; Brow et al., 2013; Knapik et al., 2020; Tentori and Richardson, 2020; Bagi et al., 2022). The TtG ratio can identify real, per cell change (or lack thereof) in microbial activity (Baelum et al., 2008; Brow et al., 2013; Tentori and Richardson, 2020) as it corrects gene transcription against the potential overestimation of activity due to growth. Other studies have used volumes of culture medium or matrix, weight/concentration of protein, or cell counts as normalizing parameters (Baelum et al., 2008; Kazy et al., 2010; Kaya et al., 2019; Michalsen et al., 2021). However, using target gene abundances for normalization is advantageous because DNA and RNA can be extracted simultaneously and both can be analyzed in one assay, keeping biases due to different techniques to a minimum.

The TtG ratio can be determined by targeted methods, particularly quantitative PCR (qPCR) that is frequently used to assess the metabolic potential (DNA-based assays) or the transcriptomic response (RNA-based assays) of specific microorganisms or microbial communities to a biogeochemical perturbation, such as a hydrocarbon contamination event by targeting characteristic functional genes (McKew et al., 2007; Yergeau et al., 2009; Genovese et al., 2014; Knapik et al., 2020; Bagi et al., 2022; Liang et al., 2023). Further, as long as the TtG ratio is normalized by a quantitative measure (i.e., qPCR data of a housekeeping gene), even transcriptomes and meta-transcriptome data can potentially inform about degradation rates. Such an approach avoids "false positives" in transcription-based prediction of cellular activity (Zhang Y. et al., 2021). It remains, however, largely unknown if the TtG ratio is a useful proxy for PAH-biodegradation activity and, by extension, crude oil degradation.

Two characteristic enzyme classes involved in PAH degradation are aromatic ring-hydroxylating dioxygenases (RHDs) and PAH hydratase-aldolases (Martin et al., 2013; LeVieux et al., 2016; Lancaster et al., 2023; Yesankar et al., 2023). RHDs are ubiquitous in PAH degraders, catalyze the first rate-limiting step in the PAH-degradation pathway, and have a very conserved reaction site. The genes encoding for these enzymes are used as a functional marker for bacterial PAH degradation in multiple habitats such as soil, freshwater, and marine sediments (Iwai et al., 2011; Meynet et al., 2015; Liang et al., 2019b). Although the diversity of studied RHD genes in marine organisms is underrepresented, a detailed characterization, including substrate specificity of nine large (alpha) and nine small (beta) RDH subunits, was performed in a Pacific Ocean isolate, *Cycloclasticus* sp. strain P1 (Wang et al., 2018). The novel gene *rhd2alpha* as well as *rhd3alpha* (previously described as *phnA1* Kasai et al., 2003; McKew et al., 2007), which are large oxygenase subunits of RHDs catalyzing the first oxidation step in phenanthrene and naphthalene degradation, respectively, were identified as key enzymes for PAH degradation in *Cycloclasticus* sp. strain P1 (Wang et al., 2018).

Polycyclic aromatic hydrocarbon hydratase-aldolases catalyze a downstream step in aerobic PAH degradation, in which analogs of trans-o-hydroxybenzylidenepyruvate are transformed to aldehydes and pyruvate (Eaton, 2000). These enzymes are less substrate specific than RHDs, and the same hydratase-aldolase is involved in both naphthalene and phenanthrene degradation in *Cycloclasticus* sp. strain P1 (Wang et al., 2018). The *pahE* gene encodes a PAH hydratase-aldolase, which was proposed as a superior marker to identify PAH-degrading bacteria in comparison to RHDs because it is well conserved among PAH-degrading bacteria (Liang et al., 2019a). Assuming *pahE* is indeed involved in both naphthalene and phenanthrene degradation in *Cycloclasticus* spp., it would be an ideal candidate for a PAH-degradation functional marker gene (Liang et al., 2022). However, it remains to be determined if the single copy genes *rhd2α*, *rhd3α*, and *pahE* in *Cycloclasticus* spp. are suitable marker genes for PAH-degradation activity, if the transcription is substrate-dependent, and if the TtG ratios can potentially inform about activity (i.e., degradation rates). Thus, it is currently unclear if these functional genes and their transcripts can be used as a proxy of PAH degradation in environmental samples.

To deduce if the transcription of functional PAH marker genes in *Cycloclasticus* spp. can inform about PAH-degradation activity,

we determined if (i) the TtG ratios of three functional PAH marker genes (i.e., *rhd2α*, *rhd3α*, and *pahE*) are correlated with the PAH-degradation rates observed in cultures of *Cycloclasticus pugetii* strain PS-1; (ii) the transcription of these marker genes in cultures of *Cycloclasticus pugetii* strain PS-1 is induced by the specific substrate (i.e., naphthalene or phenanthrene); and (iii) the genes and transcripts of these functional PAH marker genes can be found in crude oil-degrading seawater microcosms incubated under *in situ*-like conditions.

Materials and methods

Cycloclasticus pugetii strain PS-1 cultivation and PAH degradation

A freeze-dried culture of *Cycloclasticus pugetii* strain PS-1 (ATCC 51542), originally isolated from Puget Sound (Pacific Ocean) deep-sea sediments (Dyksterhouse et al., 1995), was obtained from the American culture collection (ATCC) and revived according to ATCC's instructions. Cultures were maintained on nutrient-rich, artificial seawater medium (marine broth, Difco 2216, Sigma-Aldrich, United States), supplemented either with 200 mg L^{-1} naphthalene, phenanthrene, a mixture of both PAHs, or pyruvate as carbon and energy sources.

For the experimental setup, an inoculum of *Cycloclasticus pugetii* strain PS-1 was prepared by growing the strain with pyruvate as a carbon and energy source (0.003 mol L^{-1}) in 30 mL of nutrient-rich, artificial seawater medium in serum vials (total volume 50 mL; acid rinsed, Milli-Q water rinsed, baked at 300°C for 8 h) crimped with PTFE-lined septa. From these 4-day old *Cycloclasticus pugetii* strain PS-1 pre-cultures, 300 mL of culture suspension was pooled (cell density $1.78 \times 10^7 \pm 1.18 \times 10^6$) and 1.5-mL volumes served as inocula for each of the biotic incubations for a total of 30 mL liquid in 50 mL glass serum bottles (acid rinsed, Milli-Q water rinsed, baked at 300°C for 8 h). For the PAH-containing batches, 50 µL of hydrocarbon substrates, dissolved in acetone, were added. In the cultures containing a single PAH, a final concentration of 200 mg L^{-1} naphthalene or phenanthrene was supplemented. In the cultures containing the PAH mixture, a final concentration of 100 mg L^{-1} each (both naphthalene and phenanthrene) was reached. The small amount of highly volatile acetone evaporated immediately after addition to the cultures and the PAHs were left as visible small crystals suspended in the medium. For these biotic incubations, six sacrificial, PAH-containing replicates were set up for each timepoint—one set of triplicates for PAH extraction and one set of triplicates for biological analysis. Additionally, sacrificial controls with pyruvate as a carbon equivalent to the carbon added in PAH-containing bottles (equal to pre-culture conditions) were set up in triplicates for biological analysis of DNA, transcripts, and cell numbers at timepoints of 0, 3, 6, 9, and 12 days. To evaluate potential abiotic degradation of the PAHs, non-inoculated duplicate controls were sacrificed at the first and the last day of the incubation period. All cultures were incubated in the dark on a rotary shaker at 125 rpm and at 18°C. Cell numbers, PAH concentrations, functional marker gene copies, and transcripts in the PAH-containing samples were quantified (see below) on days 0, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12.

Quantification of hydrocarbons

We quantified naphthalene and phenanthrene concentrations over time with gas chromatography coupled to mass spectrometry (GC–MS). An Agilent 6890 N GC coupled to an Agilent 7973 inert MS, equipped with an Agilent 7683 B autosampler with a J + W Scientific DB-5MS (30 m length, 0.025 mm ID, and 0.25 µm film thickness) capillary column was used. The device was operated in a single ion mode with splitless injection and a helium flow rate of 0.8 mL min^{-1}. An internal standard (D_8-naphthalene and D_{10}-phenanthrene) was added at a concentration of 20 mg L^{-1} to the respective PAH replicates and liquid–liquid extraction was performed immediately with 15 mL of cyclohexane of the whole, sacrificial vial. The samples were shaken for 30 min at 270 rpm, left undisturbed to separate the organic and the aqueous phases for a minimum of 3 days, before being diluted 1:1,000 with cyclohexane and analyzed by GC–MS. Degradation rates were calculated for each timepoint (r_t in mg L^{-1} day^{-1}) using the change in concentration between the previous (C_{t-1}) and the following (C_{t+1}) timepoint: $r_t = \left| \frac{C_{t-1} - C_{t+1}}{(t-1) - (t+1)} \right|$. This time-dependent degradation rate (in mg L^{-1} day^{-1}) was used to monitor the change in degradation over the course of the experiment.

Quantification of growth

Cell growth was quantified using two independent methods over the course of the experiments. One of these methods employed direct cell counting by taking 1 mL subsamples of culture suspension, fixing with 1% (v/v) paraformaldehyde, storing at 4°C before filtration (GTTP, 0.2 µm; Millipore), DAPI staining, and microscopic cell counts (Leica DM 5500 B epifluorescence microscope; Leica Microsystems, Wetzlar, Germany), as described previously (Rughöft et al., 2020). To compliment this method, functional marker genes were quantified as a measure of biomass, since *in silico* analysis using Geneious Prime (version 2019.1.1) and the NCBI data base (accessed in January 2019; Schoch et al., 2020) revealed that all investigated functional PAH-degrading genes were present as a single copy on the genome of *Cycloclasticus pugetii* strain PS-1 (Supplementary Table S1).

DNA and RNA extraction, processing, and quality control

To follow DNA and RNA concentrations over time, between 15 and 30 mL of culture suspension (depending on cell density) were filtered on ice through 0.22 µm Sterivex filter cartridges (Merck Millipore, Darmstadt, Germany) and immediately frozen at −80°C for subsequent analysis. To account for DNA and/or RNA losses during extraction, luciferase RNA and DNA were added to the cartridges prior to extraction as an internal standard as described by Johnson et al. (2005). Briefly, 4.47×10^5 copies μL^{-1} of luciferase DNA and 2.48×10^4 copies μL^{-1} luciferase RNA were added to each Sterivex filter after thawing and prior to extraction. The recovered luciferase DNA and RNA were quantified by performing specific qPCR assays for each sample using the primers and protocol from Johnson et al. (2005). A DNA/RNA recovery efficiency was calculated for each sample to correct for losses during sample processing. For DNA and RNA extraction, the AllPrep RNA/DNA mini kit (Quiagen, Hilden, Germany) was used following the manufacturer's instructions, but directly adding the extraction buffer to samples on the Sterivex filters. Extracted DNA was stored at −20°C and RNA at −80°C until further analysis.

To quantify transcripts, extracted RNA was purified by digesting the residual DNA with the TURBO DNA-free kit (Thermo Fisher

Scientific Inc., Waltham, Massachusetts, United States) according to the manufacturer's instructions. The purified RNA samples were transcribed to cDNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States) and quantified by qPCR (in technical triplicates) together with the corresponding DNA samples.

qPCR for functional genes

Three functional marker genes, crucial for the PAH degradation in *Cycloclasticus* spp. (Wang et al., 2018; Liang et al., 2019a), were quantified (Figure 1): *rhd3α* specific for naphthalene degradation, *rhd2α* specific for phenanthrene degradation, and *pahE* involved in the degradation of both, naphthalene and phenanthrene.

Functional maker genes and their transcripts *rhd3α*, *rhd2α,* and *pahE* were quantified using qPCR. Primers for *rhd2α* and *pahE* were designed by identifying, collecting, and aligning all the nucleotide sequences of the respective genes from *Cycloclasticus* spp. available in the NCBI database (accessed in January 2019, Supplementary Table S1) and using the primer-design tool in Geneious Prime (version 2019.1.1). For several primer candidates, qPCR protocols were developed, and the best set of primers was selected for the following analysis [Tables 1, 2, following MIQE standards (Bustin et al., 2009; Supplementary Table S2)]. For *rhd3α*, we used published primers—Cyc372F/Cyc854R—and followed an established protocol (note that in the original publication *rhd3α* is referred to as *phnA1*; Dionisi et al., 2011). Quantification of DNA and RNA via qPCR was performed in technical triplicates for each of the biological replicates, and all replicates of DNA and RNA of the same sample were quantified on the same 96 well plate. Standards and negative controls were aliquots from the same batch and quantified on each plate to ensure reproducibility. Resulting gene and transcript abundances were corrected for extraction losses based on the recovery of the added luciferase DNA and RNA in the respective sample [i.e., for normalized DNA concentration of *rhd2α* (in copies mL^{-1}) and respective recovery efficiency (in %); $\left[DNA^{norm}_{rhd\,2\alpha}\right] = \frac{\left[DNA_{rhd\,2\alpha}\right]*100}{recovery\ efficiency}$, Supplementary Figure S1].

Unitless TtG ratios were calculated by dividing transcript numbers (in copies mL^{-1}) by gene abundances (in copies mL^{-1}) to obtain a per cell measure for transcription (i.e., for *rhd2α*: $TtG_{rhd\,2\alpha} = \frac{\left[RNA_{rhd\,2\alpha}\right]}{\left[DNA_{rhd\,2\alpha}\right]}$).

To confirm the amplified sequences were not false positives, Sanger sequencing was conducted on qPCR products of randomly selected samples for each functional gene and among the different experimental conditions.

Functional TtG ratios under simulated *in situ* conditions

To supplement our pure culture findings and test the transcription of the functional marker genes under environmentally relevant conditions, we analyzed selected Arctic microcosm samples (in biological triplicates). For the microcosms, water-accommodated fractions (WAF) of crude oil, prepared as described previously (Kleindienst et al., 2015), were used as the PAH source. The amendment of WAF to seawater-containing microcosms is a commonly used method since it allows the setup and analysis of

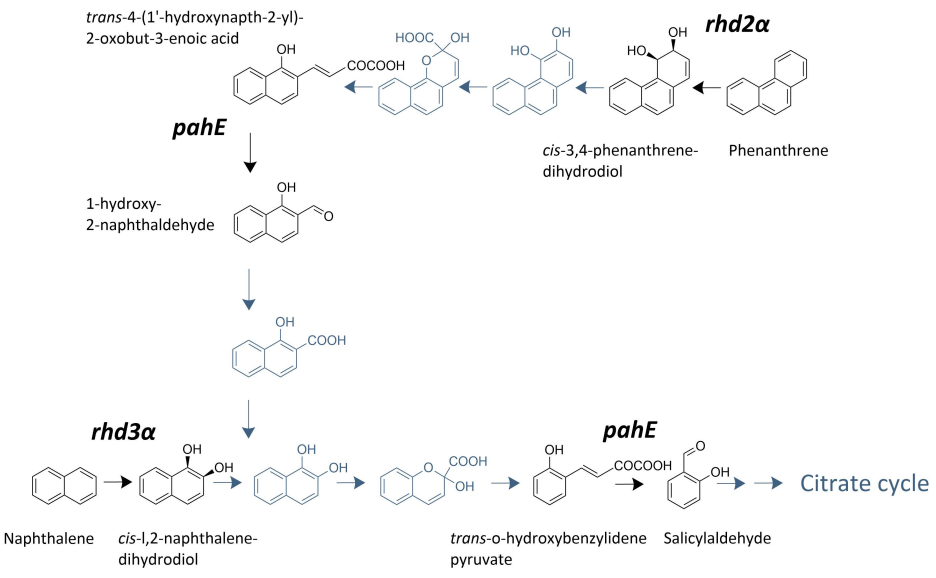

FIGURE 1
Polycyclic aromatic hydrocarbon (PAH)-degradation pathway used by *Cycloclasticus* spp. adapted from Wang et al. (2018). Steps carried out by enzymes encoded by the investigated genes are highlighted and labeled with the respective target gene.

TABLE 1 Primer characteristics for functional marker genes *rhd2α*, *rhd3α*, and *pahE*.

Target gene	Primer name	Primer sequence 5′–>3′	T_m (°C)	GC content (%)	Hairpin temp. (°C)	Primer-dimer temp. (°C)	PCR prod.
rhd2alpha[1,2]	rhd2α1126F	ACA CGA AGA GGA AAG CTG CA	59.9	50	x	1	199 bp
	rhd2α1305R	TTT TCT TGC CTG CAT AGC GC	59.8	50	42.9	x	
rhd3alpha[2,3]	rhd3α 669FD	GGG TGG ACT AGC TGG AA	54.8	59	x	3.2	120 bp
	rhd3α781RD	TTC GCA TGA ATA GCG ATG G	55.9	47	59.1	11.2	
pahE[1,4]	pahE 674F	TTG CTT GTA CAG GCC CTG AG	60	55	x	x	293 bp
	pahE 947R	CTC AGC CCA ACC TGT ACC AG	60	60	x	x	

[1]Primer design-this study, [2]gene information (Wang et al., 2018), [3]gene information (*rhd3α* = *phnA1*) and primer design from Dionisi et al. (2011), and [4]gene information (Liang et al., 2019a,b).

TABLE 2 qPCR protocols for functional marker genes *rhd2α*, *rhd3α*, and *pahE*.

	rhd2alpha[1,2]	*rhd3alpha*[2,3]	*pahE*[1,4]
SYBR green	5 µL	5 µL	5 µL
Primer F (0.5 µM)	1 µL	1 µL	0.5 µL
Primer R (0.5 µM)	1 µL	1 µ	0.5 µL
H_2O	2 µL	2 µL	3 µL
Nucleotide tmpl.	1 µL	1 µL	1 µL
Step 1	95°C, 5 min	95°C, 5 min	95°C, 5 min
	1 cycle	*1 cycle*	*1 cycle*
Steps 2–4	95°C, 20s	95°C, 20s	95°C, 20s
	62°C, 20s	62°C, 20s	58°C, 20s
		72°C, 20s	72°C, 20s
	40 cycles	*35 cycles*	*32 cycles*
Step 5	95°C, 1 min	95°C, 1 min	95°C, 1 min
	1 cycle	*1 cycle*	*1 cycle*
Step 6	62°C, 30s	62°C, 30s	62°C, 30s
	1 cycle	*1 cycle*	*1 cycle*
Step 7	62–95°C, 5 s steps	62–95°C, 5 s steps	62–95°C, 5 s steps
	melting curve	*melting curve*	*melting curve*

Volumes (10 µL in total) are given per reaction well. [1]Protocol from this study, [2]gene information (Wang et al., 2018), [3]gene information (*rhd3α* = *phnA1*) and protocol adapted from Dionisi et al. (2011), and [4]gene information (Liang et al., 2019a).

homogeneous systems to simulate oil contamination in seawater. WAF contains a range of different total petroleum hydrocarbons (TPHs) with alkanes and PAHs. For instance, a previous study found that TPHs concentration in WAF-containing microcosms were around 300 µg L^{-1} with a naphthalene concentration of around 100 µg L^{-1} (Kleindienst et al., 2015). In the present study, for preparing the WAF, 150 mL of sweet light crude oil (Danish Underground Consortium) was added to 850 mL of pasteurized Arctic seawater and mixed for 48 h in the dark at 650 rpm, and then allowed to stand for the oil and water phases to separate. The aqueous phase, constituting the WAF, was separated and added to a total amount of 900 mL Arctic seawater at two different concentrations to allow the autochthonous microbial communities—which did not contain *Cycloclasticus* spp.—to respond to the PAHs in the WAF over a period of 32 days at 1.5°C in the dark. The treatments with low-pulsed WAF received 16 pulses of 80 µL WAF equally spaced over the incubation time of 32 days. The medium-concentrated WAF microcosms received an initial one-time dosage of 800 µL WAF at day 0. Dissolved organic carbon (DOC) was quantified in technical duplicates as an estimate of organic substrate in all microcosms. Therefore, 23 mL of sample were measured using a TOC analyzer (Elementar High TOC II, Germany) in the DOC mode with thermal oxidation at 680°C and CO_2 quantification by an IR detector. To quantify gene and transcript abundances, 400 mL were filtered on ice through 0.22 µm Sterivex filter cartridges (Merck Millipore, Darmstadt, Germany) and immediately frozen at −80°C. Subsequently, DNA and RNA were extracted as described above using the AllPrep RNA/DNA mini kit (Qiagen, Hilden, Germany), the TURBO DNA-free kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States), and SuperScript III reverse transcriptase (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States). Taxonomy was determined using DNA- and RNA-based 16S rRNA (gene) amplicon sequencing as described previously (Caporaso et al., 2011). Absolute 16S rRNA gene copy numbers were quantified using a DNA-based 16S rRNA qPCR assay described previously (Lueder et al., 2022) and *Cycloclasticus* spp. absolute abundances were estimated by multiplying the DNA-based *Cycloclasticus* spp. relative abundances (based on amplicon sequencing) with the total 16S rRNA gene copy numbers (based on qPCR). For the present study, we selected those samples in which *Cycloclasticus* spp. could be detected, and we quantified the functional marker genes *rhd3α*, *rhd2α,* and *pahE* with the above-described qPCR protocols. Similar to the pure culture experiments, Sanger sequencing was used on qPCR amplicons of randomly selected DNA samples from each biological triplicate to confirm the identity of the amplicons and recognize false positives.

Statistics

All statistical tests were performed in R version 4.2.3 (2023-03-15; R Core Team, 2023) and results thereof were documented in Supplementary Table S3. Significant difference was defined when $p \leq 0.05$. When possible, ANOVA (Chambers et al., 2017) was performed with interaction term but repeated without in the case that the interaction was not significant. Following the ANOVA, *post hoc* test Tukey's 'Honest Significant Difference' (Tukey's HSD) was used (Tukey, 1949). Pearson correlation was performed with package Hmisc v5.0-1 (Harrell, 2023), and significance values were corrected for multiple testing to false discovery rate (fdr) with Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Results

Hydrocarbon degradation in axenic *Cycloclasticus pugetii* strain PS-1 cultures

To determine if the TtG ratio of the investigated functional PAH marker genes can be used as a proxy for PAH-degradation rates and if the transcription of these genes is substrate specific, pure culture experiments were performed with *Cycloclasticus pugetii* strain PS-1, grown with naphthalene, phenanthrene, a mix of both PAHs, and in a PAH-free control. Pure cultures of *Cycloclasticus pugetii* strain PS-1, previously grown in PAH-free medium with pyruvate, degraded the provided PAHs after a short lag-phase of 3 days, while no growth or degradation was observed in abiotic controls (data not shown). In the single-compound incubations (PAH provided at 200 mg L^{-1}), naphthalene was completely consumed (i.e., 86.5% between day 3 and 6; the remaining 13.5% until day 11) and naphthalene was consumed faster than phenanthrene, which was only degraded to approximately 50% after 12 days (Figure 2A). PAH concentrations differed significantly with time point ($p < 2 \times 10^{-16}$), provided substrate ($p < 2 \times 10^{-16}$), and the interaction thereof ($p = 3.58 \times 10^{-15}$), i.e., the PAH concentration changed significantly over time and dependent on the PAH addition (phenanthrene, naphthalene, or a combination of both). PAH concentrations within time points were not significantly different until day 5, but from day 6 on, phenanthrene addition retained consistently the highest PAH concentration, while naphthalene had the lowest. The combination of phenanthrene and naphthalene led to an intermediary PAH concentration that was significantly different to phenanthrene-only addition at days 6, 10, and 12, and to naphthalene-only at days 6–9 and 11 (Supplementary Table S3, Sheet A). The highest rates of naphthalene degradation of 64 mg L^{-1} day^{-1} occurred before day 5, after which the rates of degradation decreased until the naphthalene concentration dropped below 40 mg L^{-1} by day 6 (Figure 2B). Thereafter, naphthalene degradation activity remained at a constant rate of approx. 5 mg L^{-1} day^{-1} until the substrate was no longer detectable (Figure 2B). Similarly, the rate of phenanthrene degradation was higher in the beginning (33.3 mg L^{-1} day^{-1}) but then remained relatively constant for the duration of the experiment (rates of 10–18 mg L^{-1} day^{-1}; Figure 2B).

When provided with both naphthalene and phenanthrene (100 mg L^{-1} each), naphthalene was depleted rapidly to 2.1 mg L^{-1} by day 5, whereas phenanthrene degradation commenced around day 6 (Figure 2A); this was also shown in the degradation rates for the two PAHs in these incubations (Figure 2B). The degradation rate of naphthalene decreased from day 4 to 6 and was similar to that observed in the naphthalene-only experiments at an equal naphthalene concentration (51 and 1.4 mg L^{-1} day^{-1}, respectively, Figure 2B). Conversely, the rates for phenanthrene degradation were low at day 4 and 5 (rates of 2.7 and 2.9 mg L^{-1} day^{-1}) and progressively increased from day 6 onwards (Figure 2B). Following day 6, the residual naphthalene in the mixed-PAH experiment was degraded at rates of 0.1–1.4 mg L^{-1} day^{-1}, with complete degradation achieved on day 10 (Figures 2A,B). Phenanthrene degradation activity increased slowly and reached a rate comparable to the phenanthrene-only experiment at 9 days (highest rate at day 9: 23.4 mg L^{-1} day^{-1}; Figure 2B).

Growth of *Cycloclasticus pugetii* strain PS-1 and its functional gene abundance and expression

Cell counts using DAPI staining and fluorescence microscopy showed that *Cycloclasticus pugetii* strain PS-1, when supplemented with PAHs, reached stationary phase after 5–6 days [e.g., $4.74 \times 10^9 \pm 1.39 \times 10^8$ cells per mL were produced with mixed PAHs (Supplementary Figure S2)]. In the no-PAH controls supplemented with pyruvate, the cells reached stationary phase toward the end of the experiment between days 9 and 12 and $2.51 \times 10^9 \pm 3.68 \times 10^8$ cells per mL were produced (Supplementary Figure S2). The cell numbers correlated well with the copy numbers of the functional genes (Pearson's correlation coefficients 0.698, 0.735, and 0.786 for *rhd3α*, *rhd2α*, and *pahE*, respectively; $p_{adj} < 10^{-15}$, Supplementary Table S3, Sheet B, Supplementary Figure S3), as quantified via qPCR and corrected for the luciferase recovery efficiencies to adjust for extraction losses (naphthalene-only experiment shown as example, with a maximal number of $2.79 \times 10^9 \pm 8.52 \times 10^8$ cells per mL at day 12; Figure 3A). Luciferase qPCR results indicated that RNA and DNA recovery efficiencies both ranged between 3 and 30% (Supplementary Figure S1). While the total amount of extracted DNA was higher, RNA and DNA recovery efficiencies were similar, indicating an effective RNA extraction process. Transcripts of all functional genes could be quantified and were found to increase over time and bacterial growth (Figures 3B–E). The *rhd3α* transcripts were most abundant with a maximum of 10^7 transcripts per mL determined between day 10 and 12, followed by *pahE* (maximum abundance of 10^6 transcripts per mL between day 10 and 12), and *rhd2α* (maximum abundance of 10^6 transcripts per mL between day 10 and 12, Figures 3B–E). Similar results were observed in all pure culture experiments regardless of the provided substrate (naphthalene, phenanthrene, or pyruvate). Cultures amended with pyruvate reached a maximum abundance of 10^5 transcripts per mL for *rhd2α* between day 9 and 12 (Figure 3E). When normalizing the transcript copy numbers to the respective gene abundances, there was no significant change of the TtG ratios until day 9 (Figures 3B–E; Table 3), indicating per-cell expression of all investigated functional genes lacked a response to the initial PAH-degradation activity in all pure culture experiments. Although there was no initial change of the TtG ratio for all genes and treatments, the TtG ratio for *rhd3α* and *rhd2α* changed significantly over time ($p = 5 \times 10^{-5}$ and $p = 10^{-8}$, respectively) when

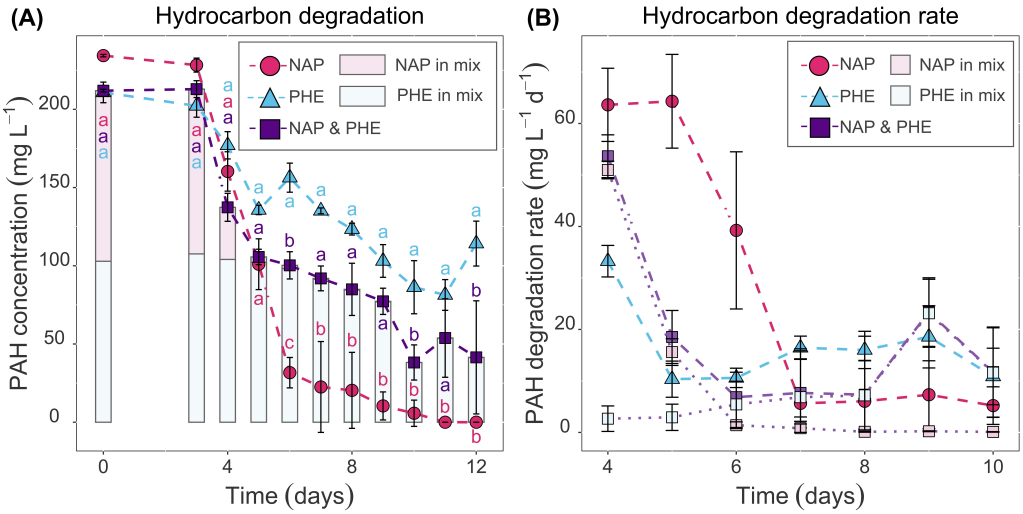

FIGURE 2
Hydrocarbon concentration quantified in sacrificial replicate serum bottles via GC–MS in mg L^{-1} **(A)** and the respective degradation rates in mg L^{-1} day^{-1} **(B)** over time. Comparison between naphthalene-and phenanthrene-only (pink circles and blue triangles, respectively) or mixed setups (purple squares represents total PAH concentration; light pink and light blue data show naphthalene and phenanthrene concentration in vials that received both PAHs). Error bars represent standard deviation. Letters indicate significant differences between PAH-amended treatments within a time point (ANOVA, *post hoc* test TukeyHSD, p_{adj}<0.05), i.e., there is significant difference when letters do not intersect.

comparing each gene separately over all provided substrates at all common measured time points (*rhd3α*: 0, 6, 9, 12 days; *rhd2α* and *pahE*: 6, 9, 12 days; Supplementary Table S3, Sheet C). Specifically, the TtG ratio at day 12 for the naphthalene-only treatment, when substrate had been completely consumed and the bacteria were starving, was significantly higher compared to earlier TtG ratios of the same treatment (Supplementary Table S3, Sheet C). For pyruvate-only amended cultures less and different time points were measured compared to the other treatments (0, 3, 6, 9, 12 days vs. 0, 4, 5, 6, 7, 8, 9, 10, 12 days), therefore, to allow more comparisons, TtG ratios were additionally compared separately for each gene and each provided substrate (Supplementary Table S3, Sheet D). The TtG ratio of *rhd3α* and *rhd2α* was constant in cultures with a combination of naphthalene and phenanthrene but varied significantly over time for naphthalene or pyruvate only (*rhd3α*: $p = 0.0001$ and $p = 0.0295$ respectively; *rhd2α*: $p = 0.0004$ and $p = 0.0422$, respectively; Supplementary Table S3, Sheet D). Furthermore, the TtG ratio for *rhd2α*, but not *rhd3α*, was significantly different over time ($p = 0.0002$) for the phenanthrene amendment (Supplementary Table S3, Sheet D). In cultures with naphthalene-only amendment, the *rhd3α* TtG ratio was significantly higher at days 10 and 12 (TtG-ratio average of both days 0.046) compared to any other time point (average of other days 0.012). The *rhd2α* TtG ratio, however, was only significantly higher at day 10 compared to any other time point (average of day 10 compared to the other days 0.007 vs. 0.001) in naphthalene-only treatments (Supplementary Table S3, Sheet D). In pyruvate amended cultures, differences were only significant between two time points each (Supplementary Table S3, Sheet D). The TtG ratio of *rhd3α* was significantly higher at 9 days (TtG-ratio average 0.044) compared to day 0 (average 0.010) and the TtG ratio of *rhd2α* was significantly higher at 12 days (average $1.7*10^{-3}$) compared to 6 days (average $6.9*10^{-4}$). The *rhd2α* TtG ratio was higher after 12 days of incubation (average 0.004) in phenanthrene-amended samples compared to any other treatments (average of other treatments average 0.001; Supplementary Table S3, Sheet D).

TtG ratios from crude oil-degrading seawater microcosms incubated under *in situ*-like conditions

To test if the genes and transcripts of the investigated functional PAH marker genes can be detected in crude oil-exposed microbial seawater communities, microcosms were set up using natural Arctic seawater and incubated under *in situ*-like conditions. To simulate crude oil contamination, the microcosms were amended with crude oil-based WAFs at two concentrations and the DOC was determined as a measure for available organic compounds. In the low WAF microcosms, DOC concentrations of 109.2 and 80.55 μM were determined at days 26 and 32, respectively. In the medium WAF microcosms, DOC concentrations of 90.63 and 90.72 μM were determined on days 26 and 32, respectively. Active *Cycloclasticus* spp. were identified in four Arctic seawater samples with absolute abundances of 1.62×10^3 and 2.89×10^4 copies mL^{-1} in low-WAF and 1.20×10^4 and 3.85×10^4 copies mL^{-1} in medium-WAF treatments, at 26 and 32 days, respectively. Although *rhd3α* and *pahE* could not be detected in samples taken from these microcosms, *rhd2α* genes and transcripts were detected and quantified in the investigated Arctic seawater microbial communities responding to WAF addition (Figure 4). Additionally, DNA and RNA were extracted from Arctic microcosm biotic controls, i.e., WAF-free treatments, and were tested with the developed qPCR assays. The target genes in these controls were below the detection limit, as expected, given that the abundance of *Cycloclasticus* spp. was also below the detection limit in these controls. WAF most likely contained a broader mixture of hydrocarbons (in addition to PAHs) and the increased transcript amount of *rhd2α* indicated phenanthrene degradation, given that *rhd2α* was shown to be essential for the initial step in this degradation pathway (Wang et al., 2018). The *rhd2α* qPCR results demonstrated that *Cycloclasticus* spp. were abundant and active as predicted from DNA-and RNA-based 16S rRNA (gene) sequencing microbial community results. Gene and transcript abundances of *rhd2α*

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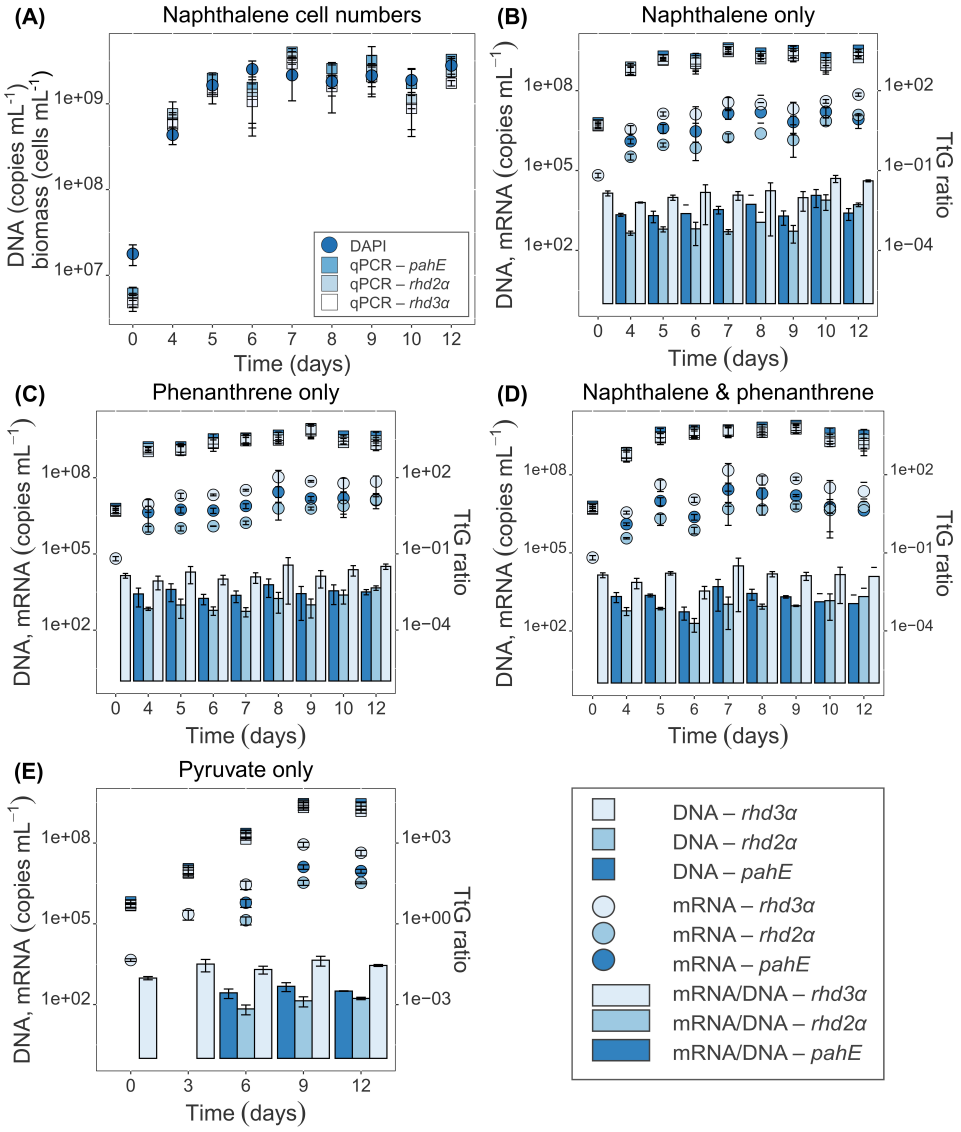

FIGURE 3
(A) Cell numbers over time for naphthalene-only experiments (all others are shown in the SI; Supplementary Figure S2). Gene-based copy numbers of *pahE*, *rhd2α*, and *rhd3α* (in copies mL^{-1}) were quantified by qPCR and corrected for losses during DNA and RNA extraction by an internal standard (Johnson et al., 2005). DAPI-stained cells were counted and given in cells mL^{-1}. **(B–E)** Target gene DNA and mRNA concentration (copies mL^{-1}) of the functional genes *pahE*, *rhd2α*, and *rhd3α* are shown in the pure culture experiments amended with naphthalene only **(B)**, phenanthrene only **(C)**, naphthalene and phenanthrene **(D)**, and pyruvate only (control; **E**); (DNA—square, mRNA—circle); TtG ratios given as bar plots. Error bars indicate standard deviation; T_0 mRNA concentration of *rhd2α* and *pahE* were below detection.

TABLE 3 Difference in TtG ratios of functional genes and transcripts in Arctic microcosm (low-WAF and medium-WAF) and pure culture experiments.

Target gene	TtG ratio in arctic microcosms		TtG ratio in *Cycloclasticus pugetii* strain PS-1			
	Experimental condition:		Growth condition:			
	WAF_{low}	WAF_{med}	NAP	PHE	NAP and PHE	PYR
rhd2α	4.61×10^{-1}	3.63×10^{-1}	2.10×10^{-3}	1.58×10^{-3}	9.92×10^{-4}	1.25×10^{-3}
rhd3α	n.a.	n.a.	1.95×10^{-2}	1.90×10^{-2}	1.43×10^{-2}	2.69×10^{-2}
pahE	n.a.	n.a.	3.94×10^{-3}	3.31×10^{-3}	2.16×10^{-3}	3.55×10^{-3}

Mean ratios calculated over all timepoints in treatments containing naphthalene (NAP), phenanthrene (PHE), both PAHs (NAP and PHE), or pyruvate (PYR) as the hydrocarbon-free control. Background color highlights the order of magnitude of the TtG ratio (from lighter to darker blue background indicating lower to higher TtG ratios).

significantly increased 4–5-fold between 26 and 32 days after WAF pulsing ($p = 0.002$ and $p = 0.001$, respectively). However, neither the amount of WAF (low vs. medium) nor the interaction between WAF and time (26 vs. 32 days) showed significant differences in gene or transcript abundance. In low-WAF microcosms, *rhd2α* gene and transcript abundances increased after WAF pulsing (between 26 and

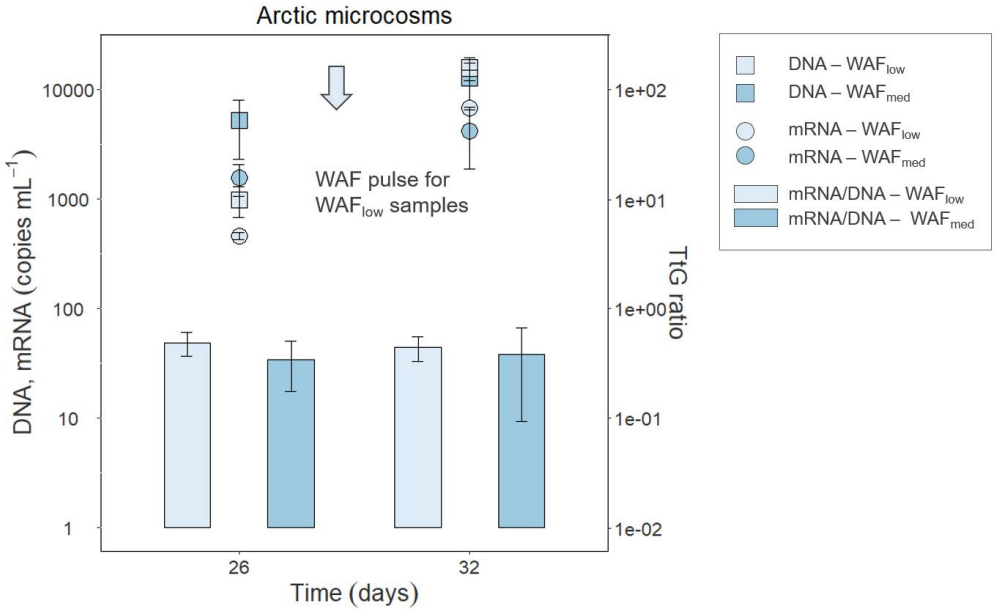

FIGURE 4
DNA and mRNA abundances (copies mL^{-1}) of the functional gene *rhd2α* and its transcripts in Arctic microcosm samples with low (frequently pulsed) or medium hydrocarbon concentrations (DNA—squares, mRNA—circles); TtG ratios are shown as bar plots. Error bars indicate standard deviation.

32 days), leading to a constant TtG ratio of $4.87\times10^{-1}\pm1.18\times10^{-1}$ and $4.44\times10^{-1}\pm1.12\times10^{-1}$ at day 26 and 32, respectively (Figure 4). In medium-WAF microcosms, *rhd2α* gene and transcript abundances also increased, but to a lesser extent compared to the low-WAF microcosms, and yet a constant TtG ratio of $3.43\times10^{-1}\pm1.66\times10^{-1}$ and $3.84\times10^{-1}\pm2.90\times10^{-1}$ at day 26 and 32, respectively, was determined (Figure 4). The TtG ratio of *rhd2α* was therefore similar (mean of $4.61\times10^{-1}\pm1.01\times10^{-1}$ and $3.63\times10^{-1}\pm2.12\times10^{-1}$ for low-WAF and medium-WAF microcosms, respectively) across time points and experimental conditions, suggesting PAH-independent expression (Table 3) and neither time nor WAF concentration had any significant impact (Supplementary Table S3, Sheet E).

Discussion

Transcriptional responses as indicator for PAH degradation

Applying the TtG ratio to Arctic seawater microcosm experiments elucidated changes in activity (i.e., transcripts) due to the growth of *Cycloclasticus* spp. (Figure 4). Between day 26 and 32, the low-WAF microcosms received a last pulse (16 pulses in total) of crude oil-derived hydrocarbons that resulted in a higher *rhd2α* transcription, which could be interpreted as increasing PAH-degradation activity in response to the substrate pulse. When considering that the hydrocarbon pulse also led to growth of *Cycloclasticus* spp.—reflected in increasing *rhd2α* gene abundance—and using the growth-corrected TtG ratio, we found that the increase in *rhd2α* transcripts was due to an increase in cell numbers (i.e., growth), rather than increased transcription and degradation activity per cell.

Moreover, our results demonstrated a lack of transcriptional response to PAH-degradation activity for all investigated marker genes in *Cycloclasticus pugetii* strain PS-1. Further studies are required to assess if PAH-independent expression of the functional genes is followed by independent expression of the encoded enzymes and constant enzyme activities. Additionally, it remains unclear if there are other PAH-degradation genes in *Cycloclasticus* spp., or even in other important PAH-degrading taxa, that can be used to estimate PAH-degradation activity. Uncorrelated expression of PAH-degradation genes to PAH degradation highlights the importance of measuring and integrating additional (bio) geochemical data (i.e., quantifying PAH rates and the extent of degradation) in order to characterize the fate of PAHs.

Influence of substrate types on PAH-degradation kinetics and marker gene expression

Cycloclasticus pugetii strain PS-1 preferred naphthalene over phenanthrene as a substrate, as indicated by higher degradation rates and preferential consumption in the mixed-PAH experiment (Figure 2). The higher degradation rate of naphthalene over phenanthrene could be explained by a difference in availability of the two substrates. One influencing factor could be the uptake of the PAHs into the cell. Current research suggests, however, that uptake of both naphthalene and phenanthrene into the cell is likely to occur via the same uptake systems (Yan and Wu, 2020). The respective dissolution kinetics, which are governed by the different physico-chemical properties of these substrates, on the other hand, can explain the difference in the availability of the two PAHs. Naphthalene is less hydrophobic, more soluble in the aqueous medium, and thus more bioavailable than phenanthrene (Yalkowsky et al., 1983; Volkering et al., 1992), which relates directly to the dissolution kinetics of these compounds, and in turn is the rate-limiting step for PAH degradation (Volkering et al., 1993; Johnsen et al., 2005). Our results demonstrated higher degradation rates for both naphthalene

and phenanthrene in the beginning of the experiments when these substrates were each added alone, and for naphthalene in the mixed-PAH experiments, followed by a decrease in the degradation rate at constant high cell densities. These results support the degradation rate being limited by dissolution kinetics. This observation can further be explained since the dissolution of organic compounds—such as PAHs—also depends on the PAH particle size (Mulder et al., 2001), with smaller particles exhibiting faster dissolution rates that are, therefore, more easily accessible. Those smaller particles were potentially degraded in the beginning, supporting growth and activity of *Cycloclasticus pugetii* strain PS-1. At higher cell numbers and with aggregation of the remaining PAH particles and cells, mass transfer from pure PAH to the dissolved fraction became rate limiting, reflected in a decreasing PAH-degradation rate (Volkering et al., 1992; Mulder et al., 2001). To further untangle the limitations due to bioavailability, the quantification of the dissolved PAH fraction—i.e., by passive samplers in the cultures combined with modeling approaches—is necessary (Ribicic et al., 2018; Vogel et al., 2023).

In the mixed-PAH experiment, *Cycloclasticus pugetii* strain PS-1 exhibited signs of diauxic metabolism of naphthalene and phenanthrene. Prior research has reported diauxic utilization of mixed carbon sources (e.g., Gorke and Stulke, 2008; Wang et al., 2019). Some studies have reported that genes involved in the degradation of the secondary (less preferred) substrate are sometimes not expressed during the time when the primary (preferred) substrate is being utilized (i.e., carbon catabolite repression; Gorke and Stulke, 2008). Diauxic growth was observed in *Cycloclasticus pugetii* stain PS-1 (Figure 2), and while the initial steps of PAH degradation are unique, the remainder of the biochemical pathway is shared (Figure 1). However, diauxic growth likely did not occur due to carbon catabolite repression in *Cycloclasticus pugetii* strain PS-1 since both *rhd2α* and *rhd3α* were expressed independent of the available substrate (naphthalene, phenanthrene, and pyruvate). These findings further support that the higher bioavailability of naphthalene led to diauxic growth.

In all pure culture incubations, the TtG ratio of *rhd3α* was approximately one magnitude higher than that of *rhd2α*, which could further explain the preference by the strain for naphthalene over phenanthrene. But assuming that the higher TtG ratio of *rhd3α* over *rhd2α* is related to an increased cellular naphthalene-degradation activity does not seem likely for three reasons. First, the number of transcript copies per cell does not inform about enzyme cellular activity and further studies would need to show that a higher TtG ratio in *rhd3α* would correspond to a higher abundance of RHD3. To date, however, no data are available comparing different RHDs in a single organism and how transcription, enzyme activities, and resulting degradation rates are linked. Additionally, both *rhd2α* and *rhd3α* in *Cycloclasticus* sp. strain P1, as well as *phnA1* in *Cycloclasticus* sp. strain A5; encode RHDs that are not entirely specific for phenanthrene or naphthalene, as they can also transform other PAHs (Kasai et al., 2003; Wang et al., 2018). When expressed in *Escherichia coli*, the RHD3-genes enabled *E. coli* to degrade 83% of naphthalene and 21% of phenanthrene, while *E.coli* with RHD2-genes degraded 90% of phenanthrene and 21% naphthalene (Wang et al., 2018). This hints at a different affinity of dioxygenases for certain substrates, rather than specificity, meaning that the dioxygenases might display a high affinity for certain substrates but do not exclusively degrade them. It is also noteworthy that *rhd3α* and *rhd2α* are co-regulated in *Cycloclasticus* sp. strain P1 by the same CRP/FNR family regulator (Wang et al., 2021). Thus, preference of naphthalene over phenanthrene due to carbon catabolite repression linked to diauxic growth seems unlikely, and co-expression of *rhd3α* and *rhd2α* is not surprising.

Although co-regulation of the *rhd2α*, *rhd3α*, and even *pahE* might led to a lack of transcriptomic responses of *Cycloclasticus pugetii* strain PS-1 in all PAH-amended experiments regardless of the provided PAH substrate, the same outcome in the no-PAH controls cannot be explained. For *pahE*, there remains uncertainty if the encoded hydratase-aldolase is specific for PAH degradation. It cannot be ruled out that *pahE* is not involved in other metabolic pathways and was therefore expressed in PAH-free controls. RHDs, however, are well studied and several reports suggest that the expression of the corresponding genes is closely linked with PAH degradation (Cebron et al., 2008; Kweon et al., 2008; Paisse et al., 2012), and therefore the detection of those transcripts in PAH-free controls is unexpected. Inoculated cells in our study could have stored PAHs from the previous subculturing step, and as such these intracellularly stored PAHs might have been metabolized in the no-PAH controls resulting in the expression of these genes. However, potential intercellular storage of PAHs does not seem likely as the pre-cultures that served as inocula were grown on pyruvate, thus eliminating any possibility of transferring intracellularly stored PAHs. Additionally, the no-PAH controls were extracted and analyzed for PAHs, using GC–MS, which included the lysis of the cells to release any possible intracellular PAHs. From this, we did not detect any quantifiable PAHs in the no-PAH controls (data not shown). We thus conclude that *pahE*, *rhd2α*, and *rhd3α* expression is unrelated to PAH-degradation rates by *Cycloclasticus pugetii* strain PS-1 and that the expression is not influenced by the substrate.

We suspect that the PAH-independent expression of functional marker genes could be due to their co-regulation with a gene encoding a downstream enzyme that is essential to central metabolism. If the expression of this gene was regulated by the same regulator as the genes that are essential for the first steps of PAH degradation, PAH-independent expression of the whole pathway/functional PAH genes would not be surprising. As well as explaining substrate-independent expression of PAH-degradation genes, we speculate that this *Cycloclasticus* strain might also use an additional, yet unknown, set of genes for PAH degradation which might be expressed in concert to these three marker genes.

Considering *Cycloclasticus* spp. are described as hydrocarbonoclastic bacteria highly adapted to PAH degradation (Yakimov et al., 2007), the enzymes for PAH degradation are an essential part of the organisms' lifestyle. This could lead to the corresponding genes, like the investigated functional marker genes, being PAH-independent or even constitutively expressed regardless of PAH availability. Indeed, the constitutive expression of hydrocarbon degradation genes was observed in other well-known hydrocarbon degrading bacteria (Cunliffe et al., 2006; Churchill et al., 2008). For example, *Mycobacterium* sp. strain CH-2 showed constitutive expression of genes encoding an alkane monooxygenase, whereas genes for PAH degradation were correlated with PAH availability and not constitutively expressed (Churchill et al., 2008). Since alkanes are presumably preferred substrates, the alkane monooxygenase might in this case be the key

enzyme for the essential lifestyle of *Mycobacterium* sp. strain CH-2. The expression of *bphC* and *xylE* in *Sphingobium yanoikuyae* strain B1, which encode enzymes involved in naphthalene degradation, were also found to be constitutively expressed (Cunliffe et al., 2006). Although it is hard to discriminate the proportion of the increase in transcription due to cellular PAH-degradation activity from the proportion due to growing cell numbers of *Sphingobium yanoikuyae* strain B1, constitutive expression of the functional genes was especially prominent when the cells were grown in carbon-and nutrient-rich LB medium. This indicates that the essential marker genes for PAH degradation might be substrate-independent or constitutively expressed when cells are metabolically active and not starving. Starvation conditions are known to influence hydrocarbon degradation activity. For example, Rughöft et al. (2020) found that starvation conditions inhibited alkane degradation of *Marinobacter* sp. strain TT1, posing the question if PAH-independent expression of *pahE*, *rhd2α*, and *rhd3α* in *Cycloclasticus* spp. can also be observed under carbon-or nutrient-limited environmental conditions.

Functional gene expression related to PAH degradation in an Arctic natural seawater community

The conclusion that transcription of the investigated functional genes alone is not a proxy for PAH-degrading activity was validated for a natural Arctic microbial community. We observed PAH-independent or possibly even constitutive expression of *rhd2α* in active (i.e., identified based on RNA) *Cycloclasticus* spp. from a natural community of Arctic seawater when amended with either low or medium concentrations of oil-derived hydrocarbons (Figure 4). PAH-independent expression of PAH-degradation genes in *Cycloclasticus* spp., therefore, was not only confirmed in carbon-rich laboratory incubations, but also suggested to occur in organisms of this genus in seawater from a natural marine environment.

We did not detect *rhd3α* and *pahE* genes or transcripts in *Cycloclasticus* spp. from the Arctic community, suggesting there is more variability in functional PAH-degradation genes in uncultivated *Cycloclasticus* spp. than expected from the similarity between the available sequenced genomes (e.g., genomes of *Cycloclasticus pugetii* strain PS-1 from this study and the well-studied *Cycloclasticus* sp. strain P1 share a pairwise nucleotide identity of 88.0%). Sanger sequencing of the amplified *rhd2α*-qPCR products from Arctic seawater samples showed 97% pairwise identity to the *rhd2α* sequence in *Cycloclasticus pugetii* strain PS-1. Although *rhd3α/phnA1* was previously used as marker gene for *Cycloclasticus* spp. in sediments (Lozada et al., 2008; Marcos et al., 2009; Dionisi et al., 2011), our findings suggest that *rhd2α* is potentially more ubiquitous in environmental *Cycloclasticus* spp. and could be a more robust marker gene to identify these organisms. What factors influence the functional variability of PAH degradation in *Cycloclasticus* spp. remains to be determined. Based on our results, comparing the isolate *Cycloclasticus pugetii* strain PS-1 to *Cycloclasticus* spp. from the Arctic microbial community, the two factors that might impact PAH-degradation rates are habitat (sediment vs. water column) and temperature (related to latitude; e.g., Gulf of Mexico vs. Arctic Ocean).

Open questions on the application of TtG ratios

Collectively, the determination of TtG ratios could be a valuable tool in various microbiology disciplines in order to assess microbial activities linked to specific functional processes. In the omics-era, powerful methods have been developed and applied broadly and across diverse scientific disciplines, including metagenomics and meta-transcriptomics, to study differential gene expression patterns in response to environmental perturbations, such as an oil spill (e.g., Mason et al., 2012; Rivers et al., 2013; Tremblay et al., 2019). Further work could potentially apply the TtG ratio as a proxy of cellular activity on the transcriptomic or even metatranscriptomic level to investigate a process-specific set of target genes and thereby increase the validity and comparability of (meta-)transcriptomic studies. Normalization of (meta-)transcriptomic data by quantitative measures of a marker gene (i.e., 16S rRNA gene or a process-specific target gene) or absolute cell counts would be necessary in the process of TtG ratio calculation (Zhang Y. et al., 2021).

So far, there are still obstacles in using the TtG ratio as a proxy for PAH biodegradation under both laboratory-based experimentation and in field studies. Substrate-independent expression of the investigated functional PAH-degradation genes (i.e., *rhd2α*, *rhd3α*, and *pahE*) indicated that TtG ratios may not be applicable to predict PAH-degradation activity in *Cycloclasticus pugetii* PS-1 nor in closely related organisms. Additionally, determining the transcription of the functional marker genes on a single-cell basis would help to exclude the possibility that the apparent PAH-independent expression was an outcome due to averaging over both active and inactive cells in a culture. Moreover, there is a knowledge gap on the preference and regulation of RHDs for microorganisms, which have multiple gene copies or alternative RHDs genes in their genome. Additionally, RHDs are multi-component enzymes—there are three to four genes required to synthesize a functional RHD—and it remains to be tested if all of these genes are PAH-independently expressed. Future work should explore if there are additional PAH-degradation genes in *Cycloclasticus* spp. that are PAH-dependently expressed and whose expression correlates with decreasing substrate concentrations during degradation. This will help to determine if using different functional marker genes, enzyme activity assays, or (quantitative) proteomic approaches are more reliable proxies for biodegradation activity. Given that obligate hydrocarbonoclastic bacteria, like *Cycloclasticus* spp., have been found in pristine environments (Gutierrez, 2019b), the substrate-independent expression of PAH-degradation genes should be further investigated in order to avoid erroneously identified PAH-degradation activities.

Conclusion

Overall, the TtG ratios of the investigated genes did not correlate with the PAH-degradation rates observed in pure cultures of *Cycloclasticus pugetii* strain PS-1. Furthermore, transcription of these marker genes in the pure cultures was not induced by a specific substrate—naphthalene, phenanthrene, or a no-PAH, carbon substrate alternative. In addition, we detected genes and transcripts of *rhd2α* in hydrocarbon-degrading Arctic seawater microbial communities during simulated oil contamination events

under *in situ*-like conditions. The TtG ratio in the Arctic seawater was also independent of the PAH-degradation activity, similar to our findings from the pure culture experiments. Overall, PAH-independent, or possibly even constitutive, expression of the investigated genes suggested *rhd2α*, *rhd3α*, and *pahE* are not suitable target genes to predict PAH-degradation activity of *Cycloclasticus* spp. through RNA-based assays. Notably, not all the investigated genes were identified in the Arctic seawater microcosms, which highlights a current knowledge gap in the functional PAH-degrading gene diversity in environmental (i.e., Arctic) *Cycloclasticus* spp. In order to achieve quantification of PAH-degradation rates *in situ*, alternative target genes need to be identified whose transcriptional responses are tightly coupled to substrate availability. Ultimately, using the correlation of the TtG ratios of process-sensitive marker genes and PAH-degradation rates would allow the robust identification of contaminated areas and associated pollutant-biodegradation processes at high spatial and temporal resolution and it could further help to mitigate hydrocarbon pollution in marine environments.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material (DataSheet ZIP); further inquiries can be directed to the corresponding author.

Author contributions

AV, KT, and SK contributed to the study conception and design. The pure culture experiment was conducted by AV. The Arctic microcosm experiment was planned by CA and SK and conducted by CA and AV. AV, KT, TG, and SK were involved in data analysis and interpretation. Statistical analysis was performed by DS. The first draft of the manuscript was written by AV and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1185619/full#supplementary-material

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Supplementing Information

TABLE S1 Gene sequence IDs of *rhd3α*, *rhd2α*, and *pahE* in *Cycloclasticus* spp. from the NCBI data base (accessed in January 2019) (Schoch et al. 2020) used for primer design. Pairwise nucleotide comparisons of target-gene sequences in the database to the respective gene sequence in *Cycloclasticus pugetii* strain PS-1 are also given.

***Cycloclasticus* spp. genome**	**Functional gene locus tag**	**Functional gene protein ID**	**Target gene**	**Pairwise identity**
C. pugetii strain PS-1	CYCPU_RS0111470	WP_016391028.1	*rhd3α*	100%
C. pugetii strain PY97N	CPC19_RS01670	WP_016391028.1	*rhd3α*	99.9%
Cycloclasticus sp. strain P1	Q91_RS11240	WP_015007028.1	*rhd3α*	99.7%
Cycloclasticus sp. strain TK8	SAMN05519226_0006	SHJ67137.1	*rhd3α*	98.3%
C. zancales strain 78-ME	CYCME_RS12395	WP_015007028.1	*rhd3α*	99.8%
C. pugetii strain PS-1	CYCPU_RS0111560	WP_015007046.1	*rhd2α*	100%
C. pugetii strain PY97N	CPC19_RS01580	WP_016390233.1	*rhd2α*	99.9%
Cycloclasticus sp. strain P1	Q91_RS11330	WP_016390233.1	*rhd2α*	98.3%
Cycloclasticus sp. strain TK8	SAMN05519226_1786	SHJ26042.1	*rhd2α*	98.3%
C. zancales strain 78-ME	CYCME_RS12485	WP_020933226.1	*rhd2α*	98.7%
C. pugetii strain PS-1	CYCPU_RS0105800	WP_015005964.1	*pahE*	100%
C. pugetii strain PY97N	CPC19_RS06820	WP_016390649.1	*pahE*	99.3%
Cycloclasticus sp. strain P1	Q91_RS05875	WP_015005964.1	*pahE*	99.6%
Cycloclasticus sp. strain TK8	SAMN05519226_2075	SHJ43395.1	*pahE*	99.1%
C. zancales strain 78-ME	CYCME_RS06220	WP_015005964.1	*pahE*	99.5%

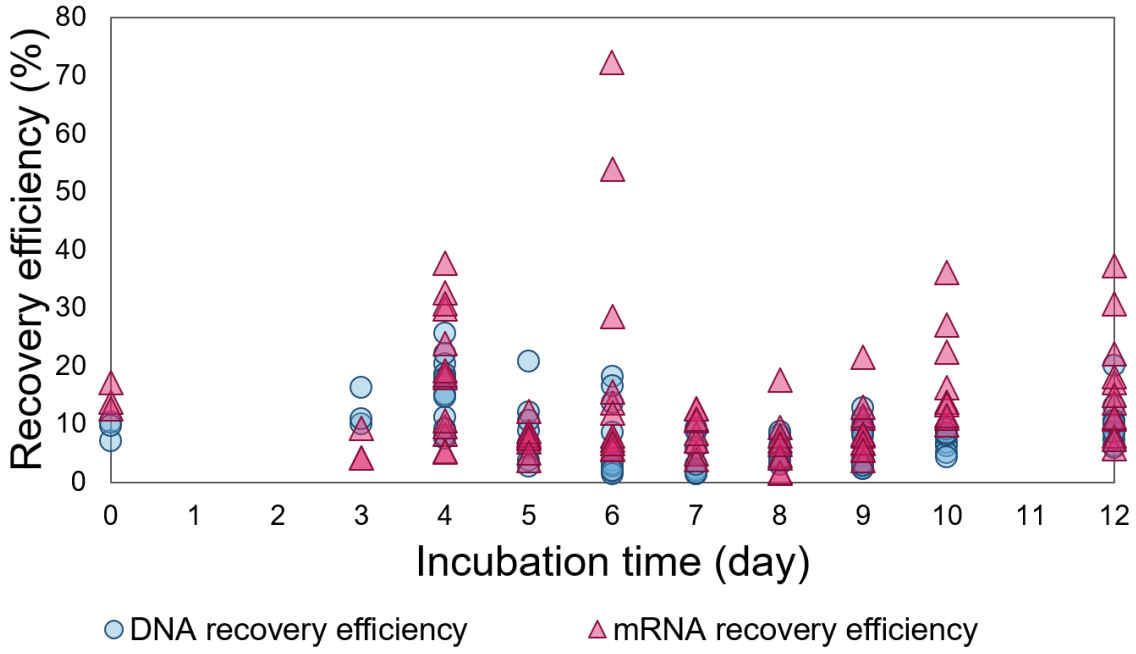

FIGURE S1 Total DNA (red circles) and total RNA (blue triangles) recovery efficiencies for all extracted samples over time. Recovery efficiencies were mostly between 3 and 30%.

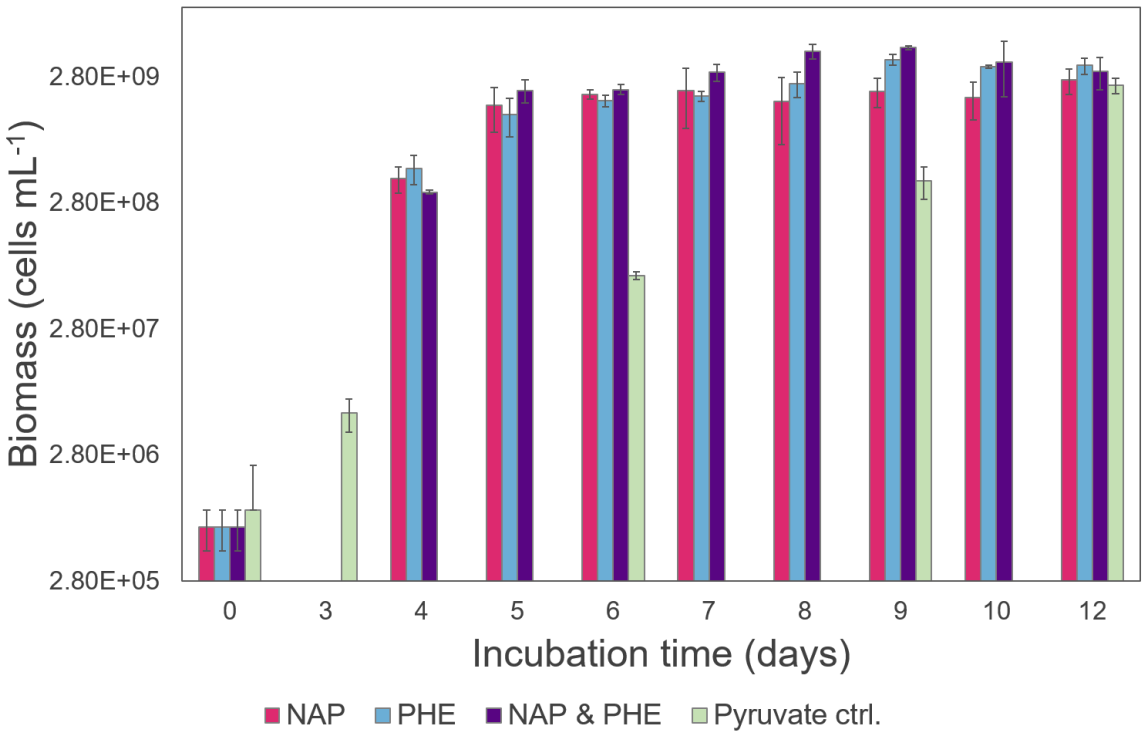

FIGURE S2 Biomass in cells mL^{-1} followed via DAPI cell counts in all experimental setups over time. Incubation conditions were naphthalene-only and phenanthrene-only (pink and blue, respectively), a mix of both PAHs (purple), and a PAH-free control, receiving pyruvate as carbon equivalent (light green). Error bars represent standard deviation between measured triplicates. At day 3, no PAH samples were measured. Pyruvate controls were sampled every third day.

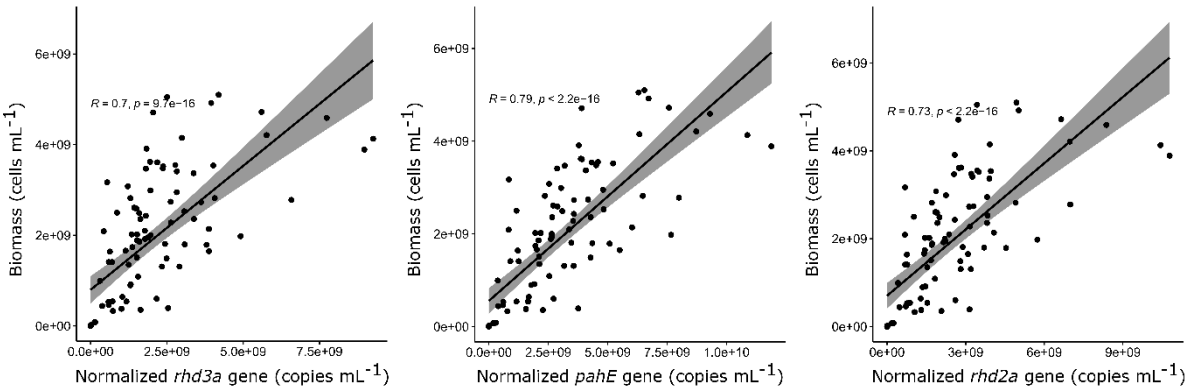

FIGURE S3 Scatter plot of biomass in cells mL^{-1} quantified by DAPI cell counts in all treatments versus the normalized gene copy number of the target genes in copies mL^{-1} (A) *rhd2α*, (B) *rhd3α* and (C) *pahE*. The linear regression line is shown with the 95% confidence interval (shaded area) including the Pearson's correlation coefficient R with the (unadjusted) p-value for each gene. Pearson's correlation analysis with adjusted p-value is also detailed in Table S3, sheet B.

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Research Article

Genetic redundancy in the naphthalene-degradation pathway of *Cycloclasticus pugetii* strain PS-1 enables response to varying substrate concentrations

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Abstract

Polycyclic aromatic hydrocarbon (PAH) contamination in marine environments range from low-diffusive inputs to high loads. The influence of PAH concentration on the expression of functional genes [e.g. those encoding ring-hydroxylating dioxygenases (RHDs)] has been overlooked in PAH biodegradation studies. However, understanding marker-gene expression under different PAH loads can help to monitor and predict bioremediation efficiency. Here, we followed the expression (via RNA sequencing) of *Cycloclasticus pugetii* strain PS-1 in cell suspension experiments under different naphthalene (100 and 30 mg L^{-1}) concentrations. We identified genes encoding previously uncharacterized RHD subunits, termed *rhdPS1α* and *rhdPS1β*, that were highly transcribed in response to naphthalene-degradation activity. Additionally, we identified six RHD subunit-encoding genes that responded to naphthalene exposure. By contrast, four RHD subunit genes were PAH-independently expressed and three other RHD subunit genes responded to naphthalene starvation. *Cycloclasticus* spp. could, therefore, use genetic redundancy in key PAH-degradation genes to react to varying PAH loads. This genetic redundancy may restrict the monitoring of environmental hydrocarbon-degradation activity using single-gene expression. For *Cycloclasticus pugetii* strain PS-1, however, the newly identified *rhdPS1α* and *rhdPS1β* genes might be potential target genes to monitor its environmental naphthalene-degradation activity.

Keywords: effect of PAH concentration; marine biodegradation; ring-hydroxylating dioxygenases; substrate-independent expression of PAH-degradation genes

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are classified as substances of concern (Environmental Protection Agency 1993) and are ubiquitous in marine environments where they bioaccumulate and are toxic to sea life as well as humans (Landrum et al. 2003, Nikolaou et al. 2009, Murawski et al. 2014, González-Gaya et al. 2016, Stading et al. 2021, Zhang et al. 2021). The biodegradation of these chemicals, principally by microorganisms, is a crucial process in their oxidation, which ultimately mitigates their toxicity effects (National Research Council 2003, Genovese et al. 2014, Duran and Cravo-Laureau 2016, Overholt et al. 2016, González-Gaya et al. 2019). Microorganisms are involved in PAH biodegradation, either by performing one step of the degradation pathway as part of a community or by using the complete pathway for the full degradation of one or more of these types of chemicals (Dombrowski et al. 2016, Joye et al. 2016, Gutierrez 2019a, Mahjoubi et al. 2021). *Cycloclasticus* spp. are known key PAH degraders commonly found in contaminated marine habitats and can completely oxidize PAHs like naphthalene, phenanthrene and pyrene (Kasai et al. 2002, Cui et al. 2014, Wang et al. 2018, Bagi et al. 2022). Naphthalene, as the PAH with the highest water solubility, is often used as a model compound in PAH biodegradation studies, and its biodegradation pathway in *Cycloclasticus* spp. has been well described (Fig. S1) (Wang et al. 2018, 2021).

To date, multiple genes involved in naphthalene degradation have been discovered and more candidate genes likely exist in the genome of the model organism *Cycloclasticus pugetii* strain PS-1. However, it is important to consider that there are still knowledge gaps in our understanding of the naphthalene- and, more generally, PAH-degradation pathways occurring in marine habitats. For example, Sieradzki et al. (2021) could not detect a complete naphthalene-degradation pathway in whole community metagenomes from PAH-contaminated surface water samples. Additionally, there is a paucity of insight into the transcriptional behavior and the factors influencing the expression of functional genes. A recent paper using metatranscriptomics revealed

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species-specific responses of two key hydrocarbon degraders, *Marinobacter* and *Colwellia*, to distinct exposure regimes resulting from additions of organic carbon derived from oil, synthetic dispersant, or oil and synthetic dispersant (Pena-Montenegro et al. 2023). In the environment, PAH emissions from anthropogenic sources range from low diffusive inputs (e.g. through transportation and river runoff) to high amounts (e.g. through shipping, oil pipelines and platform/rig accidents) (National Research Council 2003, Duran and Cravo-Laureau 2016, Ryther et al. 2021). Considering the wide range of emitted PAH loads, one factor overlooked so far in PAH biodegradation studies is the influence of environmental PAH concentrations in inducing gene expression for each of the biodegradation pathway steps.

One important application of investigating the PAH-degradation pathway is to predict and monitor a microbial ecosystem's response to PAH contamination. This response is dependent on the set of functional genes in the microbial community and the conditions that result in their expression. The genes encoding key enzymes in the PAH-degradation pathway could impact degradation differently, depending on their transcriptional behavior. Given the potential correlation between transcription and PAH-degradation activity, functional marker genes, which are transcribed dependent on available substrate, could help to develop a quantitative PCR (qPCR)-based tool (i.e. assays targeting the transcript-to-gene ratio) serving as a measure for environmental PAH-degradation activity (Wilson et al. 1999, Baelum et al. 2008, Brow et al. 2013, Tentori and Richardson 2020, Vogel et al. 2023b). However, it is unknown if there is naphthalene-concentration-dependent marker-gene expression for functional genes in *Cycloclasticus pugetii* strain PS-1.

Genes that are well conserved in PAH-degrading organisms (Meynet et al. 2015, Liang et al. 2019) can be used as marker genes to identify key PAH degraders in environmental communities through DNA-based analyses (Genovese et al. 2014, Bagi et al. 2022), even if they are transcribed independently of PAH availability. Because the expression of such substrate-independently-expressed genes does not reflect the PAH-degradation activity, transcriptomic results must be interpreted with caution. In a previous study, we found that *Cycloclasticus pugetii* strain PS-1 expressed three functional genes involved in PAH degradation independent of substrate availability (Vogel et al. 2023b); however, the transcriptional pattern of other genes of *Cycloclasticus pugetii* strain PS-1 is lacking. Furthermore, whether substrate-independent expression of functional PAH-degradation genes is a common strategy in *Cycloclasticus pugetii* strain PS-1 remains unconstrained.

Upon further examination of the genomes of *Cycloclasticus* spp., multiple genes encoding enzymes that are potentially capable of performing the same reaction in the naphthalene-degradation pathway exist, hinting at genetic redundancy (Wang et al. 2018, Bagi et al. 2022). Multiple layers of functional redundancy exist in microorganisms (Ghosh and O'Connor 2017), and while functional redundancy seemingly counters selective pressure (Nowak et al. 1997), it could lead to flexibility and thus be a benefit for the organism (Laruson et al. 2020). However, it is unknown if there is genetic redundancy in the naphthalene-degradation pathway, if there is one preferred gene or a set of genes for each step of the naphthalene-degradation pathway, and which conditions select these. In this study, we sought to determine if *Cycloclasticus pugetii* strain PS-1 possesses functional marker genes that are transcribed during naphthalene active degradation, or if substrate-independent expression of PAH-degradation genes is a common strategy in this organism. We also examined whether genetic redundancy for genes involved in the naphthalene-degradation pathway occurs in this organism.

Materials and methods

Cycloclasticus pugetii strain PS-1 cell suspension experiments

A freeze-dried culture of the well-studied PAH-degrading marine model organism *Cycloclasticus pugetii* strain PS-1 [American Type Culture Collection (ATCC) 51542], originally isolated from deep-sea sediments of the Pacific Ocean in Puget Sound (Dyksterhouse et al. 1995), was acquired from the ATCC (Virginia, USA). Strain PS-1 was revived according to the manufacturer's instructions and maintained in liquid culture with Marine-Bouillon 2216 (Sigma-Aldrich, USA), supplemented with 100 mg L^{-1} naphthalene. The cultures were confirmed as *Cycloclasticus pugetii* strain PS-1 with Sanger sequencing of the 16S rRNA gene, prior to conducting the RNA-sequencing experiment.

The experiments were conducted using a cell suspension in late log phase growth with a high cell density rather than a growing culture to eliminate growth as a parameter and thereby enable comparing the transcriptional response of *Cycloclasticus* with different PAH concentrations. To prepare inocula of cell suspensions, 700 ml of day 4 pre-culture was pooled by centrifugation (5000 xg; 10 min), and then the cell pellet was washed twice with fresh medium prior to resuspending (by vortexing) to 70 ml, resulting in a highly concentrated suspension of strain PS-1 cells. To set up the cell suspension experiment, 800 µL of the highly concentrated *Cycloclasticus pugetii* strain PS-1 inoculum was added to individual 20-ml serum vials containing a total of 8 ml of carbon- and nutrient-rich artificial seawater medium (Difco 2216, Sigma-Aldrich, USA), resulting in high cell densities of the order of 10^8 cells mL^{-1} (Fig. S2). Naphthalene was provided at two loads, 30 and 100 mg L^{-1}, which were selected to represent two concentrations from near and above the solubility of this chemical in seawater [28.96 mg L^{-1} (Vogel et al. 2023a)]. The first concentration, in low-NAP and pulse-NAP treatments, is near naphthalene's solubility in seawater and mimics diffusive low-concentration PAH input into marine environments. The added amount was expected to dissolve in the culture medium, so contaminant consumption would lead to a continuous decrease of naphthalene concentrations up to its depletion. In the pulse-NAP treatments, a second low-concentrated pulse of 30 mg L^{-1} was added at 71 h after complete degradation of the initial naphthalene. In high-NAP conditions, the addition of naphthalene above its water solubility was expected to act as a substrate reservoir. As naphthalene was consumed, more would dissolve from crystals, thus maintaining exposure of cells to relatively constant naphthalene concentrations (similar to a steady state set-up) (Vogel et al. 2023a). The reservoir was depleted once the total naphthalene concentration decreased below the water solubility limit. We considered that this experimental condition mimicked massive oil spills, which will infuse large amounts of hydrocarbons into seawater, often at concentrations above water solubility. Therefore, 53 µL, 16 µL, or two times 16 µL of a highly concentrated naphthalene stock solution (15 227.87 mg L^{-1} dissolved in acetone) were added to final concentrations of 100 mg L^{-1} (high-NAP) and 30 mg L^{-1} (low-NAP), or as two pulses of 30 mg L^{-1} (pulse-NAP), respectively. The acetone evaporated immediately, leaving the naphthalene concentration near its solubility in seawater (28.96 mg L^{-1}) and visible as undissolved crystals (Vogel et al. 2023a). The same steps were undertaken to prepare uninoculated vials with naphthalene as

abiotic controls. Naphthalene concentrations in the inoculated and uninoculated vials were quantified (described below) over the course of the experiment.

For preparation of a PAH-free control, 200 µL of 0.1 M pyruvate was added to individual 20-ml serum vials that contained 8 ml of medium. This addition was comparable with the molar amount of carbon (0.001 mol L^{-1}) added in the high-NAP set-up. All biotic vials were set up as three sacrificial samples per timepoint for naphthalene quantification, and three additional sacrificial bottles for DNA and RNA extraction, which were all incubated in the dark on a rotary shaker (125 r/m; 18°C). Sacrificial bottles were used to avoid mass losses of naphthalene due to volatilization. A liquid-liquid extraction of each bottle, with the strong solvent cyclohexane, was used to fully extract the total naphthalene from both the sorbed phase and the aqueous phase. Naphthalene was quantified after inoculation, as well as after 12, 24, 48 and 168 h for high-concentration treatments, and once in the PAH-free controls after 24 h. PAHs were quantified in low-concentration treatments after inoculation and after 12, 24 and 71 h. Pulsed treatments had identical conditions to low-NAP treatments between 0 and 71 h and, therefore, were only sampled after 73, 85, 97 and 168 h. Samples for RNA-sequencing were taken 2 h after inoculation for all naphthalene-containing treatments, including following the second pulse of naphthalene in the pulse-NAP set-ups, and at a later nutrient-depleted ("starvation") timepoint when naphthalene was fully degraded.

Quantification of hydrocarbons

Naphthalene concentrations were quantified by gas chromatography coupled to mass spectrometry (GC-MS). A liquid-liquid extraction with cyclohexane of each complete sacrificial bottle was performed to avoid mass losses of naphthalene due to volatilization. As an internal standard, 12.6 mg L^{-1} of D_8-naphthalene (dissolved in acetone) was added into the crimped vials before using 10 ml of cyclohexane as an organic solvent to extract naphthalene. Subsequently, the samples were shaken for 35 min at 270 r/m and the organic and aqueous phases were allowed to separate undisturbed for 48 h in the dark. The organic phase was subsequently extracted using a glass syringe, and diluted 1:100 with cyclohexane, before naphthalene was quantified using an Agilent 6890 N GC coupled to an Agilent 7973 inert MS. The GC-MS was equipped with an Agilent 7683 B autosampler with a J + W Scientific DB-5MS capillary column (30-m length; 0.025-mm ID; 0.25-µm film thickness) and was operated in single ion mode with splitless injection and a helium flow rate of 0.8 ml min^{-1}.

DNA and RNA extraction and processing

For quantification of the transcription of PAH-related genes during active naphthalene degradation (2 h after addition of naphthalene) and under starving naphthalene conditions, RNA sequencing was conducted. The timepoints for sampling were chosen based on previous experiments (Vogel et al. 2023b). Hence, for DNA and RNA analyses, the total volume (8 ml) of each sacrificial bottle was filtered through sterile 0.22-µm Sterivex filters (Merck Millipore, Darmstadt, Germany) on ice and stored at -80°C until further processing. DNA and RNA were extracted using the Allprep mRNA/DNA kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions with the exception that the extraction buffer was added directly to the Sterivex cartridges, and these were then vortexed at medium power for 4 min before removing the buffer with a 10-ml syringe and transferring it to the first spin column. Immediately upon completing these extraction steps, DNA was stored at -20°C and mRNA at -80°C until further analysis. For RNA purification, the TURBO DNA-free kit (Thermo Fisher Scientific Inc., USA) was used to digest any remaining DNA, following the manufacturer's instructions. The resulting DNA-free RNA was submitted to the Institute for Medical Microbiology and Hygiene (University of Tübingen) for library preparation using Illumina stranded RNA prep, rRNA depletion with Ribo-zero Plus, and sequencing using NextSeq 500 High Output Kit v2.5 (75 cycles, Illumina, San Diego, CA, USA).

Monitoring cell numbers—qPCR of functional genes *rhd3α* and *rhd2α*

To ensure the cell density remained constant over the course of the cell suspension experiment, we followed two functional genes—*rhd3α* and *rhd2α*—using qPCR. Both genes encode alpha subunits of aromatic ring-hydroxylating dioxygenases (RHD2*α* and RHD3*α*) and are present only once per genome in *Cycloclasticus pugetii* strain PS-1 (Vogel et al. 2023b). Both qPCR methods and primers were already developed and used elsewhere: *rhd3α* by Dionisi et al. (2011) (note that the gene is referred to as *phnA1* in this study) and *rhd2α* in our previous study (Vogel et al. 2023b). Primer and qPCR protocol information can be found in Vogel et al. (2023b) (see also Tables S1 and S2).

Bioinformatic analysis

To analyze the RNA-sequence data, an index database, adjusted to small genomes (genomeSAindexNbases 4), was created with Spliced Transcripts Alignment to a Reference (STAR) v2.6.1d (Dobin et al. 2013) using the *Cycloclasticus pugetii* PS-1 genome assembly ASM38441v1 from NCBI (RefSeq assembly accession GCF_000384415.1). Based on the created reference genome index, nf-core/rnaseq v1.4.2 (https://nf-co.re/rnaseq) (Ewels et al. 2020), and its containerized software, was used with singularity v3.4.1 (Kurtzer et al. 2017) and executed with Nextflow v21.10.3 (Di Tommaso et al. 2017). Nf-core/rnaseq performed quality checks using FastQC v0.11.8, a quality control tool for high-throughput sequence data that is available online at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Andrews 2010). Additionally, <0.5% of basepairs per sample were removed due to adapter contamination and trimming of low-quality regions with Trim Galore! V0.6.4. Up to 11.4% to 35.4% rRNA sequences were removed with SortMeRNA v2.1b (Kopylova et al. 2012). Using STAR v2.6.1d, 81.38% to 95.62% reads were aligned, and, finally, 6.0 to 12.3 million counts per sample (total: 225 986 447; average: 9 416 101 reads) were assigned to genes by featureCounts v1.6.4 (Liao et al. 2014).

To compare the differences in total gene expression between the different set-ups, multidimensional scaling (MDS) was conducted. MDS based on expression profile distances of the top 500 log2-fold changes between sample pairs with edgeR v3.26.5 was plotted for all treatments (Robinson et al. 2010). To assess the differences in the transcription levels of each gene, gene counts were used in differential abundance analysis for all treatments in R v4.1.1 (2021–08–10) with DESeq2 v1.34.0 (Love et al. 2014) using singularity container https://depot.galaxyproject.org/singularity/bioconductor-deseq2:1.34.0--r41h399db7b_0. Significant differences were postulated for transcripts using the Benjamini and Hochberg-adjusted *P*-value (P_{adj}) ≤ 0.05. Finally, gene counts were transformed to transcript per million (TPM) to allow for the comparison of gene expression between treatments with StringTie2 v2.1.7 (Kovaka et al. 2019).

To identify key metabolic functions in the genome of strain PS-1, functional hidden Markov model profile-based KEGG orthology (KO) annotation and KEGG mapping (Kanehisa and Goto 2000, Kanehisa et al. 2016a,b) was conducted using KofamKOALA v2022-06-02 (Kanehisa et al. 2016a,b, Aramaki et al. 2020) with a threshold E-value = 0.01, with release 102.0 (https://www.genome.jp/tools/kofamkoala/). All genes were grouped by functional categories using the KEGG database "modules" (level 3) while omitting any unannotated genes, and cumulative mean TPM of the biological triplicates were plotted per functional category.

Identification of the genes involved in PAH degradation and defining transcription categories

To find candidate genes for all the reactions involved in the naphthalene-degradation pathway, we compiled a database containing 154 *Cycloclasticus pugetii* strain PS-1 genes related to PAH degradation (Table S3, sheet A) using the annotations of the NCBI database (August 2022) (Schoch et al. 2020), the KEGG database (August 2022) (Kanehisa and Goto 2000, Kanehisa et al. 2016a,b) and published literature (Wang et al. 1996, Kasai et al. 2003, Wang et al. 2018, Liang et al. 2019, Wang et al. 2021, Bagi et al. 2022).

A sub-set of PAH-related genes was generated from the curated 154 PAH-gene literature-database (described above; Table S3, sheet A) using R (v3.6.0 (2019–04–26) and Rstudio 2022.07.2+576). Genes of interest were selected based on high expression under naphthalene-containing conditions (mean TPM in at least one of the naphthalene-containing treatments within the 90th percentile, mean TPM ≥ 451). The resulting 43 genes (Table S3, sheet B) were assigned to the categories defined below, sorted by pathway step, and the TPMs, as a measure of expression, were plotted in a heatmap. Furthermore, the significance between the naphthalene-containing conditions and the no-PAH control was highlighted (genes with -1 < log2-fold change < 1 and $P_{adj} \leq 0.05$).

To identify patterns in gene expression pertaining to naphthalene availability, four categories based on the genes' transcription in the presence or absence of naphthalene (NAP_{pos}, NAP_{neg}, NAP_{indep}, no pattern) were defined (Table S4), as follows: (i) significant upregulation under naphthalene-containing conditions and/or downregulation under naphthalene-starvation conditions compared with the no-PAH control (NAP_{pos}); (ii) significant downregulation in naphthalene-containing conditions and/or upregulation under naphthalene-starvation conditions compared with the no-PAH control (NAP_{neg}); (iii) genes that showed no significant upregulation or downregulation in the naphthalene-containing treatments (NAP_{indep}) compared with the no-PAH control; and (iv) genes with no clear pattern in upregulation or downregulation, irrespective of the naphthalene concentration (no pattern).

Results

Hydrocarbon degradation

Naphthalene was fully degraded, regardless of the starting concentration, by cell suspensions of *Cycloclasticus pugetii* strain PS-1 over 168 h (Fig. 1), while cell numbers remained constant—between 1.28×10^8 and 4.66×10^8 cells L^{-1}—over the course of the experiment (Fig. S2). In high-NAP treatments, degradation of 103.2 ± 0.93 mg L^{-1} naphthalene to 1.12 ± 0.70 mg L^{-1} was observed within 48 h. A maximum degradation rate of 4.16 mg L^{-1} h^{-1} was reached within the first 12 h, followed by a decrease in degradation activity between 12 and 48 h (rates for each 12-h interval were 1.28 and 1.53 mg L^{-1} h^{-1}, respectively). The residual naphthalene was fully degraded during the remaining incubation time. In both low-NAP (30.4 ± 0.58 mg L^{-1}) and pulse-NAP (26.73 ± 0.33 mg L^{-1}) treatments, the complete degradation of naphthalene occurred within 12 h of inoculation and after pulsing, with a degradation rate of 2.51 and 2.23 mg L^{-1} h, respectively. Naphthalene concentrations in all abiotic controls remained constant over the course of the experiment and no naphthalene was detected in PAH-free controls (Table S5).

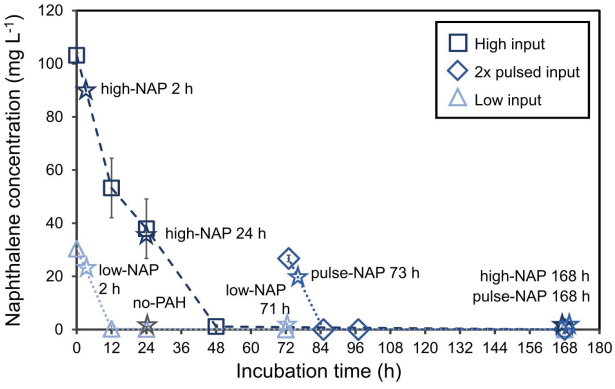

Figure 1. Naphthalene concentration in mg L^{-1} over time, quantified by GC-MS. Squares show concentrations in high-NAP (100 mg L^{-1}), lighter blue triangles illustrate concentrations in low-NAP (one dosage; 30 mg L^{-1}) and darker blue diamonds give the concentrations in the pulse-NAP treatments (2×30 mg L^{-1}). Pulsed treatments were treated equally to low-NAP treatments up until 71 h and, therefore, only sampled after 73, 85, 97 and 168 h. Error bars represent the standard deviation of the respective three sacrificial samples and are sometimes within the marker. Timepoints for RNA-sequencing are marked with stars and colored according to treatments.

Overall transcriptional activity in *Cycloclasticus pugetii* strain PS-1

To study gene expression profiles, we assessed similarities in log2-fold changes between samples. MDS between gene expression profiles showed that the transcription intensity within treatments (conducted in biological triplicates) was much more similar than between treatments (Fig. S3). Naphthalene concentration in the samples and the time elapsed following the addition of PAH could explain the difference in transcription between the samples. Correspondingly, transcription in all samples where naphthalene had been completely consumed (starvation) was similar, regardless of the initial substrate concentration. The 2-h low-NAP and pulse-NAP treatment transcriptomes also grouped together and the high-NAP samples after 2 h grouped separately from them (Fig. S3).

To identify key metabolic functions, transcripts were grouped by functional categories. Among all transcripts, expression of genes related to aromatic hydrocarbon degradation was the highest of all KEGG modules (level 3) across treatments (even in the no-PAH control), confirming that PAH degradation is an important metabolic feature of *Cycloclasticus pugetii* strain PS-1 (Fig. 2). Notably, only 39 genes were annotated in the KEGG database as related to PAH degradation. However, we identified a set of 154 genes potentially involved in PAH degradation by using available databases and literature, indicating that there might be a larger group of genes related to PAH degradation, and the analysis based on KEGG modules might be underestimating the true activity of PAH-related genes in strain PS-1.

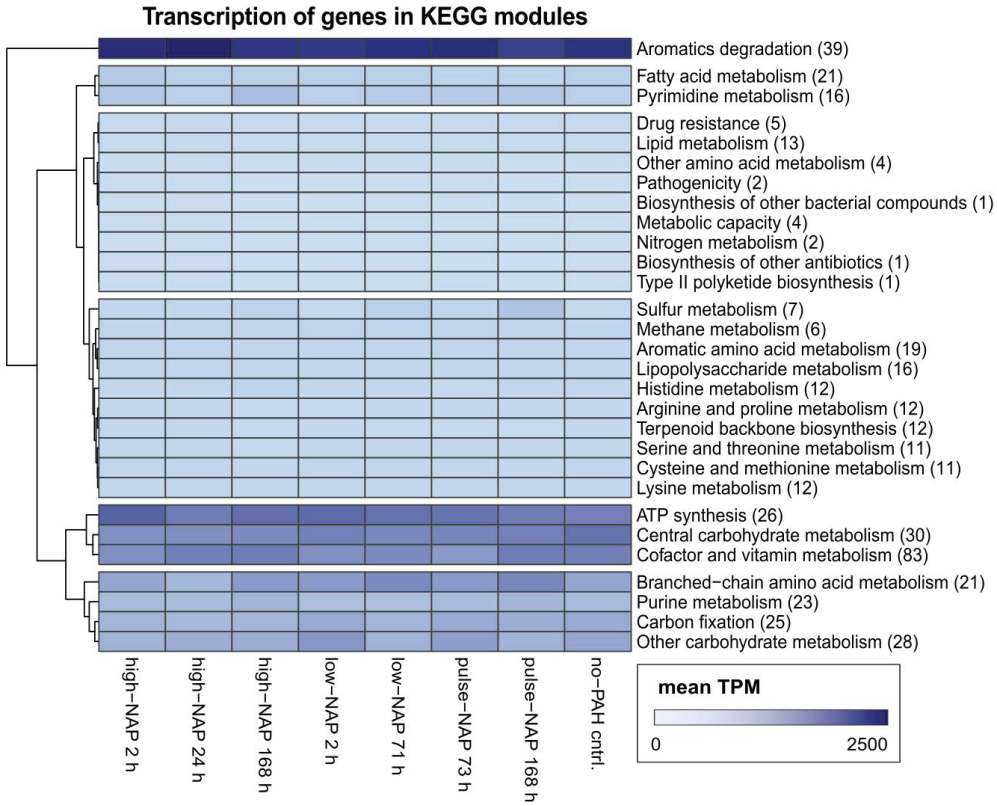

Figure 2. Expression per set of genes accumulated in KEGG modules (level 3). The expression is given in mean TPM per biological triplicate: 0 (white) to 2500 (dark blue). The number of genes in each functional category is shown in brackets. High and low concentration experiments received 100 and 30 mg L^{-1} of naphthalene at T_0, respectively, whereas the pulse treatments received 30 mg L^{-1} at T_0 and after 71 h. Transcription was determined after 2, 24 and 168 h for high-NAP treatments, after 2 and 71 h for low-NAP treatments (i.e. right before pulsing) and after 73 and 168 h for pulse-NAP treatments. PAH-free controls with pyruvate as carbon equivalent were analyzed after 24 h (no-PAH).

Transcriptional response of genes on the PAH-gene cluster

To further explore the transcriptional activity of *Cycloclasticus pugetii* strain PS-1 during PAH degradation, we examined the expression of a cluster of 33 genes, some of which were annotated in KEGG and all of which were previously identified as PAH-degradation genes in closely related *Cycloclasticus* spp. (Fig. 3, Table S3, sheet C, and Table S6) (Kasai et al. 2003, Wang et al. 2018). This gene cluster was previously described as "cluster E" and was differentially expressed in strain P1 grown on PAHs (naphthalene, phenanthrene and pyrene) compared with acetate-grown cells (Wang et al. 2018). Part of the cluster was published in 2003 as "*phn*A-cluster" (locus tag CYCPU_RS0111430 to CYCPU_RS0111480 in Table S6) and was described as enabling *Cycloclasticus* sp. strain A5 to degrade PAHs such as naphthalene and phenanthrene (Kasai et al. 2003). In the present study, the genes in the expression heatmap (Fig. 3) were sorted by pathway step of naphthalene degradation and significant transcription was highlighted [genes with $-1 <$ log2-fold change < 1 and adjusted *P*-value (P_{adj}) ≤ 0.05].

The full PAH-gene cluster in strain PS-1 contains nine genes that encode RHD subunits, including *rhd2α* and *rhd3α* [also referred to as *phnA1a* in previous publications (Kasai et al. 2003, McKew et al. 2007, Dionisi et al. 2011)]. The RHD subunits were identified as functional marker genes for PAH degradation, encoding enzymes that catalyze the first step of phenanthrene and naphthalene degradation, respectively (Wang et al. 2018). Further, CYCPU_RS0111455 was identified as putatively encoding an additional RHD β subunit based on its homology to other RHDs and the functional prediction of its active site (Paysan-Lafosse et al. 2023). Additional genes implicated in the PAH-degradation pathway were also identified in the gene cluster [e.g. a regulator factor (*dpr2*) (Wang et al. 2021)].

We used the significance (P_{adj}) and expression (log2-fold) change to define categories based on the transcriptional response of a gene to the presence or absence of naphthalene. Of the 33 genes in the PAH cluster, five genes—four encoding RHDs and one for a putative RHD—were assigned to the NAP_{pos} category, meaning they were significantly upregulated in naphthalene-containing treatments and/or downregulated under naphthalene-starvation conditions compared with the no-PAH control. Conversely, three RHD-encoding genes were assigned to the NAP_{neg} category, demonstrating their lack of transcriptional upregulation in the presence of naphthalene and/or their upregulation under naphthalene-starvation conditions. A further 19 genes, like *rhd3α*, were naphthalene-independently expressed (NAP_{indep} category), and their transcription did not change over time, even in the presence of different naphthalene concentrations. Overall, only five of the 33 genes had low expression values, with a TPM below the 50th percentile (Fig. 3, percentile definition see Fig. S4), demonstrating the high expression trend of genes within the PAH cluster in strain PS-1. More importantly, the expression of another five genes was between the 90th and 95th percentile (mean TPM over all naphthalene-containing treatments between 451 and 878), and six were in the 95th percentile (mean TPM over all naphthalene-containing treatments equal and above 878), indicating very high expression (Fig. 3). These very highly expressed genes were annotated as encoding the small and large subunits of a RHD (CYCPU_RS0111490 and CYCPU_RS0111495,

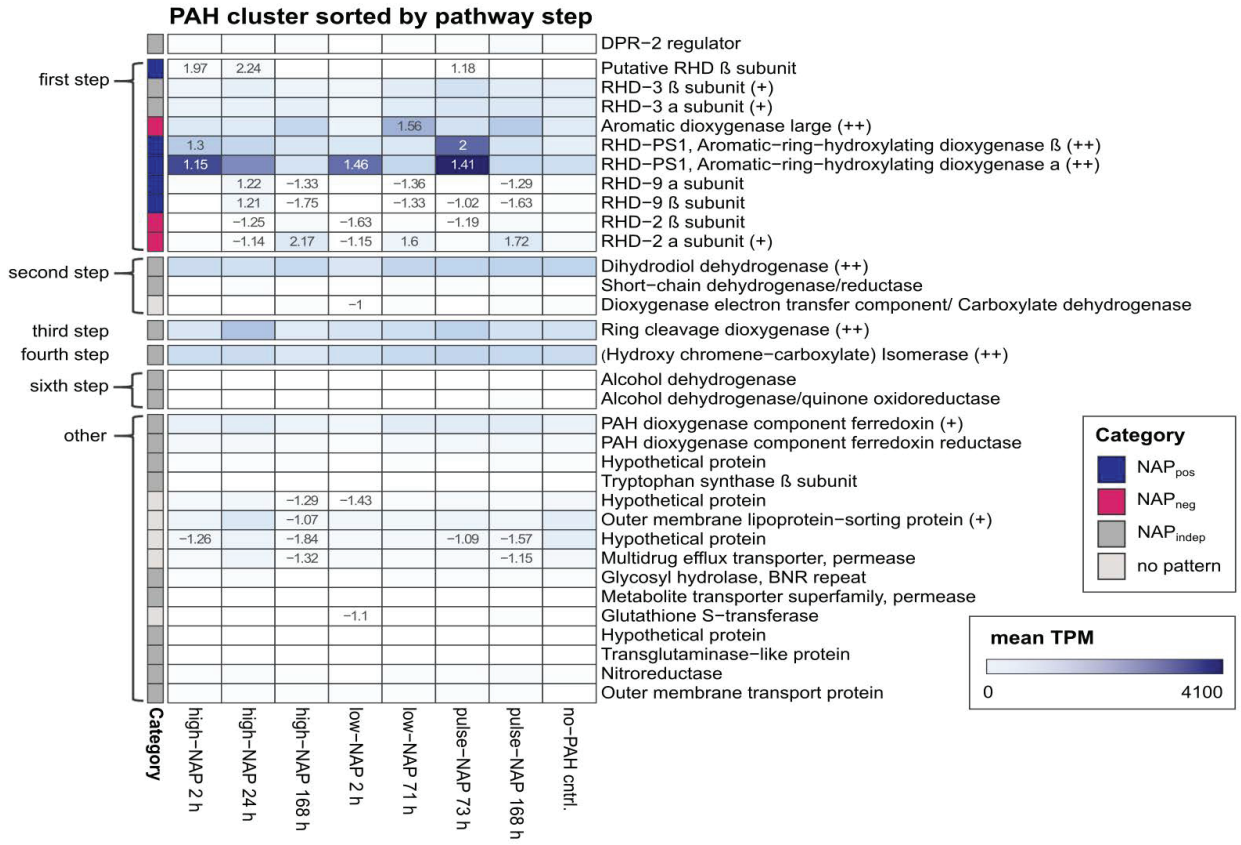

Figure 3. Expression in PAH cluster sorted by pathway step. Heatmap showing the expression (mean TPM per biological triplicate for each treatment) of genes associated with PAH degradation in the PAH cluster. Genes are sorted by pathway step (regulator, first step, second step, third step, fourth step, sixth step and other). Significant log2-fold changes to the no-PAH control are displayed ($P_{adj} \leq 0.05$, $1 <$ log2-fold change > 1), 90th $\leq$ mean $TPM_{NAP} <$ 95th percentile (+), and mean $TPM_{NAP} \geq$ 95th percentile (++) are indicated. High and low concentration experiments received 100 and 30 mg L^{-1} of naphthalene, respectively, at T_0, whereas the pulse treatments received 30 mg L^{-1} at T_0 and after 71 h. Transcription was determined after 2, 24 and 168 h for high-NAP treatments, after 2 and 71 h for low-NAP treatments (right before pulsing) and after 73 and 168 h for pulse-NAP treatments. PAH-free controls with pyruvate as carbon equivalent were measured after 24 h.

respectively), a dihydrodiol dehydrogenase/4-hydroxythreonine-4-phosphate dehydrogenase (CYCPU_RS0111480, second step of naphthalene degradation), a ring-cleavage dioxygenase (*rcd*) (CYCPU_RS0111460, third step of naphthalene degradation) and an (hydroxychromene-carboxylate) isomerase [CYCPU_RS0111430, fourth step of naphthalene degradation (Wang et al. 2018)]. The exceptionally highly expressed genes CYCPU_RS0111490 and CYCPU_RS0111495 are annotated to encode beta and alpha subunits of a RHD in the PAH cluster (Wang et al. 2018). However, the role of this RHD in naphthalene degradation is unknown as it remained untested in *Cycloclasticus* sp. strain P1 (Wang et al. 2018) and is uncharacterized in all other isolated *Cycloclasticus* sp. We, therefore, tentatively named them *rhdPS1β* and *rhdPS1α*, respectively. Using the basic local alignment search tool—BLAST (Altschul et al. 1990, Zhang et al. 2000, Morgulis et al. 2008)—we confirmed that the genes with the highest nucleotide pairwise identity were aromatic ring-hydroxylating dioxygenase subunits alpha and beta from *Cycloclasticus* sp. strain P1 and *Cycloclasticus zancles* 78-ME with 98.01% and 99.79% for *rhdPS1α* and *rhdPS1β*, respectively. Protein functional analysis with InterPro (Paysan-Lafosse et al. 2023) confirmed that the genes encoded RHD alpha and beta subunits and that *rhdPS1α* contains the Rieske [2Fe-2S] iron-sulphur domain. In the KO database (Kanehisa and Goto 2000, Kanehisa et al. 2016a,b), *rhdPS1α* and *rhdPS1β* are listed (#K16320) as involved in aminobenzoate degradation. Current literature has investigated potentially similar genes in a *Sphingomonas* sp. and a *Burkholderia* sp. (Chang et al. 2003, Gai et al. 2010); however, the respective genes are not highly related: the nucleotide pairwise identities were 48.1% and 55.5% for *rhdPS1α* and 49.3% and 50.2% for *rhdPS1β*, respectively. The transcription of two other RHD-encoding genes in the PAH cluster (*rhd9α*—CYCPU_RS0111505 and *rhd9β*—CYCPU_RS0111510) responded positively to naphthalene availability. The mean TPM over all naphthalene-containing treatments for *rhd9α* and *rhd9β*, however, was between the 50th and 75th percentile, indicating lower expression and a potentially lesser role during naphthalene degradation. Two additional genes encoding for RHD subunits (*rhd3α*—CYCPU_RS0111470 and *rhd3β*—CYCPU_RS0111465) were expressed independently of naphthalene availability, while three genes (*rhd2α*—CYCPU_RS0111555, *rhd2β*—CYCPU_RS0111560 and *phnA1b*—CYCPU_RS0111475) were assigned to the NAP_{neg} category.

We identified genes encoding all the enzymes prior to step 6 of the naphthalene-degradation pathway (Wang et al. 2018), except for a hydratase aldolase, which is necessary for the fifth step. However, the two genes encoding for two alcohol dehydrogenases (sixth step) were not highly expressed (i.e. CYCPU_RS0111520 mean $TPM_{NAP} \leq$ 50th percentile and CYCPU_RS0111545 mean TPM_{NAP} between the 50th and 75th percentile), indicating that some PAH-related genes used in naphthalene degradation by strain PS-1 may be located elsewhere in the genome.

From further analysis of the significantly upregulated and downregulated genes between the high-NAP-2 h and the no-PAH control, we identified an additional 184 genes that were differen-

tially expressed (Fig. S5). In total, 23 of those genes (Fig. S5–marked as black stars) were found in the 154 PAH-related gene-database compiled from the literature (Table S3, sheet A), and only four of these 23 were part of the previously described PAH cluster (Fig. 3, Table S3, sheet C, and Table S6), indicating that manual curation of genes involved in PAH degradation is essential.

Transcription of genes highly involved in PAH degradation

To identify genes for all the reactions actively involved in the naphthalene-degradation pathway, a subset of genes that were highly expressed in the presence of naphthalene (i.e. TPM ≥ 90th percentile in at least one of the naphthalene-containing treatments) was selected from the curated literature database, containing 154 PAH-related genes, and further analyzed (Table S3, sheets A and B). Out of this subset of 43 selected genes (Fig. 4, Table S3, sheet B), 16 genes were already known as part of the previously described PAH cluster (Fig. S6). From these 43 genes, 12 fell into the NAP_{pos} category (Fig. S6) and eight of those were annotated as encoding for RHD subunits. The only two genes in the NAP_{neg} category (both already known from the PAH cluster, see Fig. S6) also encoded RHD subunits. Most of the remaining genes were expressed independently of naphthalene concentration (20 genes of the selection, three of them encoding RHD subunits, Fig. S6), while the final nine genes showed no clear naphthalene-related pattern of expression. Further genes were potentially relevant for the strain due to their high expression (15 genes—90th ≤ mean TPM_{NAP} < 95th percentile) or very high expression (10 genes—mean TPM_{NAP} ≥ 95th percentile). Although 14 genes, which were not part of the PAH cluster, were identified as highly expressed (90th ≤ mean TPM_{NAP} < 95th percentile) and four as very highly expressed (mean TPM_{NAP} ≥ 95th percentile), the genes with the highest TPM values were still *rhdPS1α*, followed by *rhdPS1β*, which fell into the NAP_{pos} category. Additionally, a gene encoding a hydratase aldolase (CYCPU_RS0105800) that could potentially conduct the fifth step in the naphthalene-degradation pathway (Fig. S1) had very high expression and was in the NAP_{pos} category. Overall, 25 out of the selected 43 genes could be assigned to one of the steps from 1 to 7 (Fig. S1) in the naphthalene-degradation pathway, and 14 of those genes coded for RHD subunits, potentially involved in the initial step of the degradation pathway (Fig. 4).

Discussion

Naphthalene-dependent transcription of functional marker genes

We identified genes encoding an uncharacterized RHD alpha and beta subunit (termed *rhdPS1α* and *rhdPS1β*) whose expression responded significantly to naphthalene (Fig. S1). Although located in the previously described PAH cluster, as illustrated in Fig. 5 (Kasai et al. 2003, Wang et al. 2018), RHD-PS1 has not been characterized in any *Cycloclasticus* species to date. Comparing the transcription with the expression of other RHD-encoding genes, the substantial expression of *rhdPS1α* and *rhdPS1β* in response to naphthalene availability and degradation activity of *Cycloclasticus pugetii* strain PS-1 suggests that the RHD-PS1 dominates the first step of naphthalene degradation. The newly described genes are, therefore, promising candidates for functional marker genes and could potentially be used for monitoring the naphthalene-degradation activity of strain PS-1 with a qPCR-based method that quantifies the transcript-to-gene ratio (Baelum et al. 2008, Brow et al. 2013, Tentori and Richardson 2020, Vogel et al. 2023b), and could ultimately help to track PAH-degradation activity in contaminated environments. Knockout mutant (Perez-Pantoja et al. 2009) as well as recombinant-protein-expression studies (Wang et al. 2018) would help to further determine the role of *rhdPS1α* and *rhdPS1β* in the naphthalene-degradation pathway of *Cycloclasticus pugetii* strain PS-1.

Additionally, we identified four genes encoding RHD alpha and beta subunits that responded positively to naphthalene input and are likely involved in the first step of naphthalene degradation (Fig. 4). Two of the genes—*rhd7α* (CYCPU_RS0104890) and *rhd7β* (CYCPU_RS_0 104 895)—were highly expressed (90th ≤ mean TPM_{NAP} < 95th percentile) and the resulting RHD-7 was previously described as an enzyme that initializes fluoranthene degradation in *Cycloclasticus* sp. strain P1 (Wang et al. 2018). Further studies are needed to confirm the role of these other NAP_{pos} RHDs in naphthalene degradation, however, the observed link between transcription and naphthalene-degradation activity indicates they may play a key role in *Cycloclasticus pugetii* strain PS-1. The highly, and naphthalene-dependent, expressed genes might enable strain PS-1 to rapidly increase the number of enzymes (e.g. RHD-PS1 and RHD-7) and thereby quickly adapt to acute naphthalene contamination.

The transcription of three additional RHD-encoding genes among the PAH cluster (Fig. 5) depended significantly on the available naphthalene concentration. However, the genes were downregulated in the presence and upregulated in the absence of naphthalene, so they were attributed to the NAP_{neg} category. While *rhd2β* was highly expressed (90th ≤ mean TPM_{NAP} < 95th percentile) in the absence of naphthalene, we observed the substrate-independent transcription of *rhd2α* in previous growth experiments with naphthalene and phenanthrene (Vogel et al. 2023b). This could indicate that transcription of *rhd2α* and *rhd2β* are not only dependent on the availability of naphthalene, but also the cultivation conditions of the organism (growth experiment vs. cell-suspension experiment) or as a response to starvation (Vogel et al. 2023b). Notably, these RHD-encoding genes might exhibit a different transcriptional behavior for alternative PAHs and further studies should examine the transcription of the identified marker genes in response to other PAH substrates such as phenanthrene, biphenyl and naphthalene derivates.

Substrate-independent transcription of PAH genes

No significant upregulation in naphthalene-rich and/or downregulation under naphthalene-starvation conditions compared with the no-PAH controls indicated transcription independent of naphthalene availability (NAP_{indep} category).

Two of those genes were encoding the ferredoxin and ferredoxin reductase (part of the PAH cluster, Fig. 5), which are important parts of the multicomponent RHD enzymes, and thus, relevant in the first step of PAH degradation. However, the resulting components are often shared between different RHD enzymes (Wang et al. 2018). Given this, it is not surprising to find the genes as constitutively or naphthalene-independently expressed in an organism that has highly expressed genes for at least six different RHDs.

Out of 13 genes potentially encoding enzymes involved in the naphthalene-degradation steps 2 to 6 (Fig. S1)—from the entire genome—10 were naphthalene-independently expressed, indicating that substrate-independent expression of genes in the PAH degradation pathway occurs regularly in strain PS-1. Although all

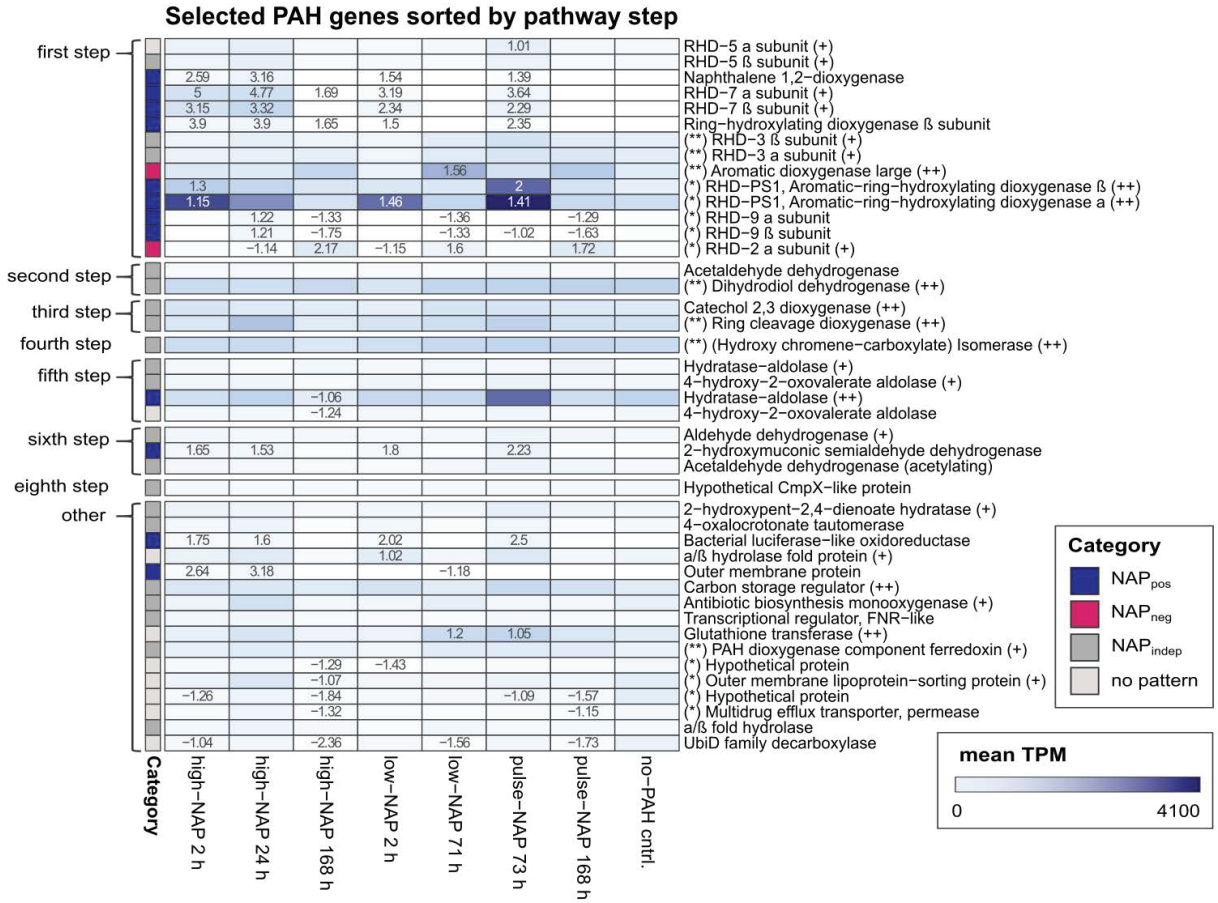

Figure 4. Selected, highly expressed PAH degradation genes. Heatmap showing the expression: mean TPM from 0 (white) to 4100 (dark blue) per biological triplicate. Genes were selected if TPM was within the 90th percentile in at least one naphthalene-containing treatment. 90th ≤ mean TPM_{NAP} < 95th percentile (+), and mean TPM_{NAP} ≥ 95th percentile (++) are indicated. Significant log2-fold changes compared with the control (pyr.24 h) are displayed ($P_{adj} \leq 0.05$, 1 < log2-fold change > 1). Genes are sorted by pathway step (from top to bottom: first step, second step, third step, fourth step, fifth step, sixth step, eighth step and others). High- and low-concentration experiments received 100 and 30 mg L^{-1} of naphthalene, respectively, at T_0, whereas the pulse treatments received 30 mg L^{-1} at T_0 and after 71 h. Transcription was determined after 2, 24 and 168 h for high-NAP treatments, after 2 and 71 h for low-NAP treatments (i.e. right before pulsing), as well as at 73 and 168 h for pulse-NAP treatments. PAH-free controls with pyruvate as carbon equivalent were measured at 24 h. Genes that are part of the PAH-cluster are indicated [W Wang et al. (2018) (∗), Kasai et al. (2003) (∗∗)].

the genes involved in the downstream steps are likely associated with PAH degradation, some of them could produce enzymes that are involved in other pathways (Hernaez et al. 2002). Overall, it remains uncertain if the isomerases, dehydrogenases and hydratase aldolases are specific for PAH degradation. The non-exclusive use of the downstream enzymes in other metabolic pathways might explain the naphthalene-independent expression of some of the PAH-degradation genes; however, further investigation would be required to prove this hypothesis.

RHDs, however, are specific for the first step in PAH degradation (Gibson and Parales 2000, Singleton et al. 2012, Yesankar et al. 2023). Finding three genes encoding RHD subunits that are highly expressed (90th ≤ mean TPM_{NAP} < 95th percentile) and naphthalene independent is, therefore, surprising. The genes *rhd3α*, *rhd3β* and *rhd5β* all fell into the NAP_{indep} category, and while their highly expressed nature makes them potentially important for strain PS-1, their transcription is likely not a response to acute naphthalene input. These results were corroborated in previous qPCR-based experiments where substrate-independent transcription was observed for *rhd3α* as well as for *pahE* (a hydratase aldolase; CYCPU_RS0105800) in *Cycloclasticus pugetii* strain PS-1 (Vogel et al. 2023b).

The reasons for substrate-independent transcription of PAH-degradation genes, especially RHDs, are unknown. Given that PAH degradation is central to the metabolism of *Cycloclasticus pugetii* strain PS-1, as shown by analyzing the transcription per functional category (Fig. 2), the corresponding enzymes might be essential for the lifestyle of this highly specialized organism. Constitutive—i.e. substrate-independent—expression of functional genes has been observed in other hydrocarbon-degrading bacteria (Cunliffe et al. 2006, Churchill et al. 2008), making it a potentially common—but overlooked—phenomenon. Further, substrate-independent transcription of RHD-encoding genes could have practical reasons for this organism. Due to the PAH-independent expression of *rhd3α*, *rhd3β*, *rhd5β* and potentially *rhd5α*, RHD-3 and RHD-5 might be used as a "background" naphthalene-degradation system for strain PS-1. The PAH-independent expression should allow a constant availability of these enzymes, which might lead to substrate-independent PAH-degradation capacity.

Genetic redundancy in the PAH-degradation pathway of *Cycloclasticus pugetii* strain PS-1

Genetic redundancy is a common phenomenon in all pathway steps, but particularly in the first step of naphthalene degradation. Five RHDs (RHD-PS1, RHD-7, RHD-3, RHD-5 and RHD-2) were identified, for which transcription of at least one subunit-encoding gene was within the 90th percentile.

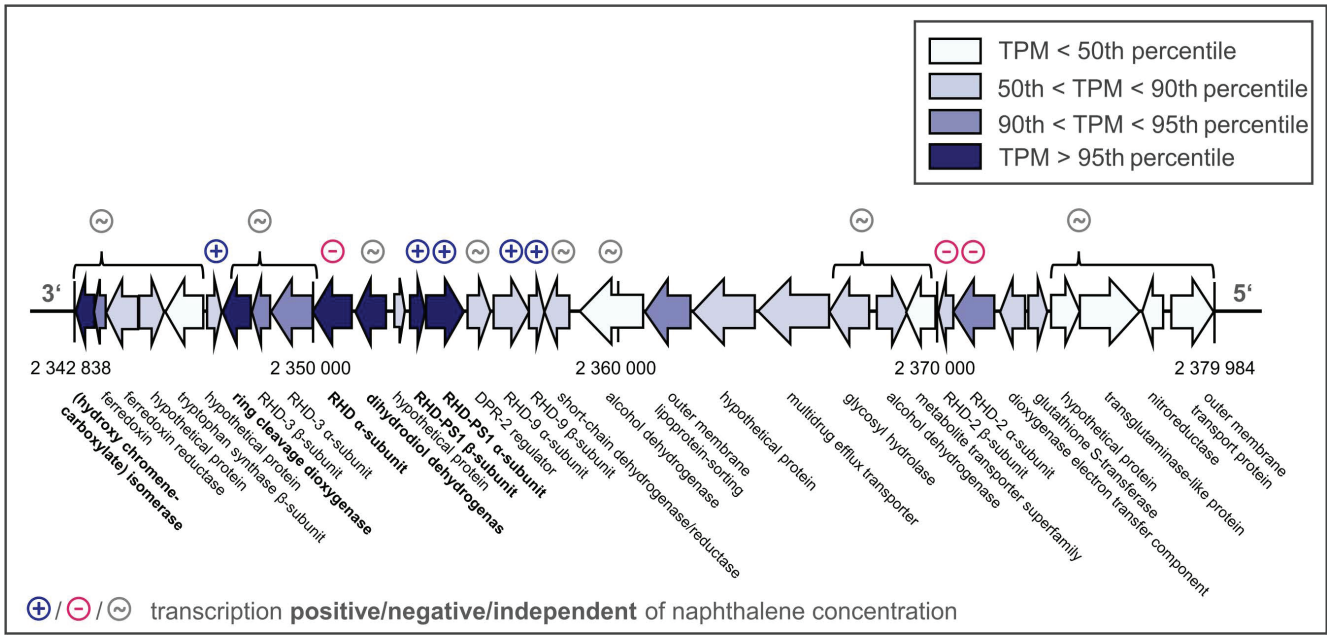

Figure 5. Gene cluster of PAH degradation-related genes within the *Cycloclasticus pugetii* PS-1 genome. Further information on the genes is provided in Table S3, sheet C, and Table S6; the cluster was previously described in closely related *Cycloclasticus* sp. strains A5 and P1 (Kasai et al. 2003, W Wang et al. 2018). Coloring indicates the percentile of expression level by mean TPM values over all naphthalene-containing experiments in our study (Fig. S4). Color coding for naphthalene-concentration dependence: blue, NAP_{pos}; pink, NAP_{neg}; gray, NAP_{indep}. PAH cluster spanning 37 146 bp; total length of the genome is 2 383 924 bp.

The reasons for genetic redundancy in PAH-degradation genes, especially in genes encoding RHDs, are unknown. RHDs catalyze the first reaction, which is the rate-limiting step in PAH-degradation, so the rate of carbon and energy gain increases with an accelerated rate of the first step. Potentially, genetic redundancy of RHD-encoding genes could be beneficial for strain PS-1 by providing several enzymes for the same function and, thereby, increasing the rate of the first step in naphthalene degradation (Perez-Pantoja et al. 2009). Additionally, a fast consumption of naphthalene should lead to a steeper gradient between the bioavailable dissolved naphthalene and the pure compound. The steeper gradient in turn would accelerate the dissolution rate of naphthalene (Volkering et al. 1992, 1993, Vogel et al. 2023a), leading to a faster substrate supply rate for the organism.

Genetic redundancy of RHD-encoding genes could, alternatively, indicate a highly specialized PAH-degradation strategy of *Cycloclasticus pugetii* strain PS-1. Potentially, two PAH-degradation enzymatic systems could be used, depending on the availability of PAHs: a steadily available "background" system and a specialized "rapid-response" system for acute PAH input. The "background" system would include RHDs that were encoded by substrate-independently expressed genes that would be constantly available and could degrade chronic PAH contamination at trace amounts. Considering that most genes encoding these naphthalene-degradation-pathway enzymes were expressed independently of naphthalene addition (NAP_{indep} category), it is reasonable to assume that the substrate-independent expression of highly relevant PAH-degradation genes is the default strategy for *Cycloclasticus pugetii* strain SP-1. Moreover, all of the pathway steps other than the first step were shared for multiple PAHs (i.e. naphthalene, phenanthrene and pyrene) in a closely related *Cycloclasticus* sp. (Wang et al. 2018), which emphasizes how essential those genes are in this potential "background" system. The "rapid-response" system, on the other hand, would include (mainly) RHDs that were upregulated and downregulated depending on the availability of substrates and would be expressed in the case of a high-contamination event to quickly degrade substrates at high concentrations. This system would enable *Cycloclasticus pugetii* strain PS-1 to respond quickly and benefit from the sudden availability of high loads of substrate. Moreover, the enzymes encoded by genes in this system might be more substrate specific because they potentially only need to respond to one or two PAHs and convert them at high rates. RHD-3 and RHD-5 would, therefore, potentially serve as a "background" metabolism, which could be active in naphthalene- or PAH-free environments, whereas RHD-PS1 and RHD-7 might be responding to acute naphthalene input.

Cycloclasticus spp. in the environment

The hypothesized PAH-degradation strategy of *Cycloclasticus pugetii* strain PS-1 offers several potential benefits to the growth of *Cycloclasticus* spp. in the environment. Environmentally occurring concentrations are much lower than those routinely used in the laboratory [e.g. the sum of dissolved concentrations of 64 PAHs in surface water samples was between 2 ng L^{-1} (Indian Ocean) and 3.5 ng L^{-1} (North Atlantic) (González-Gaya et al. 2016)]. Consequently, we hypothesize that *Cycloclasticus* spp., as part of a native community, are commonly operating with the "background" system and high-substrate affinity system of enzymes, for which the genes are substrate-independently expressed. Conversely, the "rapid-response" system, with enzymes encoded by PAH-dependently expressed genes, is likely triggered by higher substrate concentrations and consists of low-substrate affinity enzymes.

We propose that the "rapid response" system would be triggered by a naphthalene concentration lower than that of naphthalene solubility in seawater (28.96 mg L^{-1}) given that environmental concentrations of naphthalene are, in reported cases, much lower than 28.96 mg L^{-1} (Diercks et al. 2010, González-Gaya et al. 2016, Vogel et al. 2023a). This "rapid response" en-

zymatic system could, for example, potentially have operated in the contaminated hydrocarbon plume that formed after the Deepwater horizon oil spill, where PAH concentrations amounted to a maximal 189 μg L^{-1} (Diercks et al. 2010). Changing the PAH/naphthalene input and thereby substrate availability, hence, is an important environmental condition that can influence the PAH/naphthalene-degradation rate considerably (Mostafa et al. 2019, Bacosa et al. 2021). Further studies should investigate the threshold concentrations required to activate the "rapid response" system.

Identifying genes of both enzymatic systems in our model organism *Cycloclasticus pugetii* strain PS-1 highlights the level of adaption to a PAH-degradation lifestyle. Considering *Cycloclasticus pugetii* strain PS-1 was isolated from Puget sound (Dyksterhouse et al. 1995)—a habitat with many natural oil seeps—a high level of adaption to both chronic traces of PAH, as well as recurring high-input PAH pollution, is not surprising. We anticipate that *Cycloclasticus* spp. can outcompete other PAH-degrading bacteria that only have one step or a subset of steps in the PAH-degradation pathway. The advantage of the *Cycloclasticus* spp. would arise from them having multiple genes for each step of the pathway and hypothetically a "rapid-response" system of (mainly) RHDs (Sieradzki et al. 2021). The substrate-dependent transcription of (mainly) RHDs might give *Cycloclasticus pugetii* strain PS-1 the possibility to rapidly increase its degradation rate, which could be one explanation why *Cycloclasticus* spp. are detected ubiquitously and can be isolated from PAH-amended enrichment cultures (Wang et al. 2008, Gutierrez et al. 2013, Cui et al. 2014, Rizzo et al. 2019).

Environmental implications

With the increasing amount of environmental metagenomic and metatranscriptomic studies, it is important to consider what the detection of genes and transcripts from the NAP_{pos} and NAP_{indep} categories would imply for the respective microbial communities and environments. The detection of genes from the NAP_{indep} category, such as *rhd3α* and *rhd3β*, or genes encoding downstream enzymes, such as *pahE*, could be—given that the genes are truly PAH-pathway specific—used as marker genes for PAH-degrading organisms, as previously suggested (Dionisi et al. 2011, Liang et al. 2019). The transcription of those genes, however, does not imply that the organisms are actively degrading PAHs, but rather indicates a general metabolic activity. Further, an environment with microbial community members possessing NAP_{indep} genes may not always be pristine (Gutierrez 2019b), even if no apparent PAH contamination is detectable (Angelova et al. 2021). We, therefore, posit that some environments may be continually purged from constant trace inputs of PAHs without enriching PAH degraders or inducing PAH-degrading pathways, given that a "background" system of genes is constantly transcribed.

By contrast, genes from the NAP_{pos} category, especially *rhdPS1α* and *rhdPS1β*, could potentially not only identify PAH-degrading organisms, but also be used as functional marker genes for high naphthalene-degradation activity of *Cycloclasticus pugetii* strain PS-1. Determining the transcript-to-gene ratio of NAP_{pos} genes of a microbial community could be a valuable tool to quantify the cell-number-independent degradation rate of specific compounds and, thereby, assess the PAH-biodegradation performance after high-concentration contamination events, like oil spills or in a laboratory experiment (Wilson et al. 1999, Baelum et al. 2008, Brow et al. 2013, Tentori and Richardson 2020, Vogel et al. 2023b). Further, identifying organisms with NAP_{pos} genes in an environmental community could indicate a faster environmental recovery from acute high-input contamination.

Nonetheless, genetic redundancy in key genes for PAH degradation makes it difficult to quantify the PAH-degradation activity in environments based on the expression of a single gene or set of genes. Detailed knowledge about the PAH-degrading community and the categorization of the involved PAH-degradation genes would be necessary to select a suitable set of target genes for the investigated environment. Moreover, the expression of such identified target genes could vary between strains and could be sensitive to other environmental factors. Further studies like a global assessment and characterization of RHDs in all currently available metagenomes and transcriptomes are necessary before a robust set of genes for the quantification of *in situ* PAH-degradation rates can be proposed.

Open questions on genetic redundancy in PAH degradation

Several open questions remain regarding genetic redundancy in *Cycloclasticus pugetii* strain PS-1. Further studies, including knockout mutants and enzymatic assays, are required to investigate for molecular redundancy (Perez-Pantonja et al. 2009), identify which of the RHDs have an affinity for other PAHs, or to confirm these RHDs are performing the initial step in naphthalene degradation (Wang et al. 2018). Additional research is necessary to determine whether the expressed RHD-encoding genes are induced by naphthalene, but the corresponding enzymes are not produced and/or not used in naphthalene degradation.

Moreover, in a closely related *Cycloclasticus* sp., the genes encoding RHD-2, RHD-3 and five other genes associated with the PAH-degradation pathway were co-regulated by the same regulator (Table S6) (Wang et al. 2021). In *Cycloclasticus pugetii* strain PS-1, however, these genes were expressed differently: some NAP_{neg} and others were substrate independent. Our understanding of conditions and substrates influencing the regulation of PAH-degradation genes in *Cycloclasticus* spp. remains undefined. Further studies are needed to determine if an alternative regulation mechanism is used in *Cycloclasticus pugetii* strain PS-1, or if the genes are co-regulated but the mRNA of the apparent NAP_{neg} genes is potentially degraded and, therefore, not substrate-independently detected.

Because the model organism in this study is an isolated and very well studied *Cycloclasticus* sp., further studies could investigate if the observed transcriptional patterns changed in the case when *Cycloclasticus pugetii* strain PS-1 would live as part of a PAH-degrading community. Further, Arctic *Cycloclasticus* spp. from a natural community were shown to have a different set of RHDs than expected from the genomes of the isolated *Cycloclasticus* spp. (Vogel et al. 2023b). Investigating the transcription of the PAH genes in environmental *Cycloclasticus* spp. under PAH-free or even hydrocarbon-free conditions—given that some *Cycloclasticus* spp. can degrade alkanes (Rubin-Blum et al. 2017, Gutierrez et al. 2018)—would be the next step. Because the cell numbers of *Cycloclasticus* spp. under hydrocarbon-free conditions are typically low and mostly not detectable, assessing the transcription of PAH-degradation genes will be challenging. Therefore, quantifying transcripts (through qPCR) or conducting metatranscriptomic studies following an environmental contamination event when all hydrocarbons are consumed, similar to our starvation conditions, could elucidate the role of *Cycloclasticus* spp. in an environmental microbial community.

Conclusion

The naphthalene-dependent transcription of multiple RHDs indicated that strain PS-1 is very well adapted to respond instantly to high-concentration inputs of PAHs. This fast reaction can potentially be achieved by increasing the overall degradation rate through the maintenance of an enzymatic "rapid response" system. Currently, it is not possible to deduce the degraded PAH or the environmental degradation activity by targeting the transcription of a single functional gene or set of genes. The newly described functional marker genes *rhdPS1α* and *rhdPS1β*, however, are promising target-gene candidates to quantify naphthalene-degradation activities through DNA/RNA-based methods, because their transcription seems to correlate to naphthalene degradation in *Cycloclasticus pugetii* strain PS-1. Using these genes, the monitoring of PAH degradation could, in future, be conducted in a high-throughput manner by using molecular-based methods such as the TtG ratio. This in turn could facilitate more efficient PAH bioremediation because the measures or conditions could be adapted more rapidly when the monitoring proves that the degradation rate is changing. Further, an additional set of PAH-degradation genes that were expressed independently of naphthalene was also identified. Those genes are involved in all reactions of the currently known naphthalene-degradation pathway in *Cycloclasticus* spp., indicating there might be another set of PAH-degrading enzymes that is potentially used as a "background" system for the degradation of environmentally occurring trace amounts of PAHs.

The observed genetic redundancy in PAH-related genes—particularly RHDs—along the naphthalene-degradation pathway and their varying levels of transcription under different conditions has not been reported previously and should be further studied. This genetic flexibility indicated by the hypothesized two enzymatic systems could enable PAH degraders to respond to fluctuating hydrocarbon inputs in a need-based way. Understanding the degradation pathway used by key PAH degraders under varying conditions, such as low vs. high PAH concentrations, is important to assess contamination scenarios correctly. These assessments could be used to enhance bacterial PAH degradation in, for example, bioremediation scenarios.

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Author contributions

Anjela L. Vogel (Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft), Katharine J. Thompson (Conceptualization, Supervision, Validation, Writing – review & editing), Daniel Straub (Data curation, Formal analysis, Methodology, Validation, Writing – review & editing), Florin Musat (Validation, Writing – review & editing), Tony Gutierrez (Validation, Writing – review & editing), and Sara Kleindienst (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing)

Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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Data availability

The raw RNA-sequencing data from this study have been deposited with links to the BioProject accession number PRJNA838751 into the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA838751).

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Genetic redundancy in the naphthalene-degradation pathway of *Cycloclasticus pugetii* strain PS-1 enables response to varying substrate concentrations

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Supporting Information

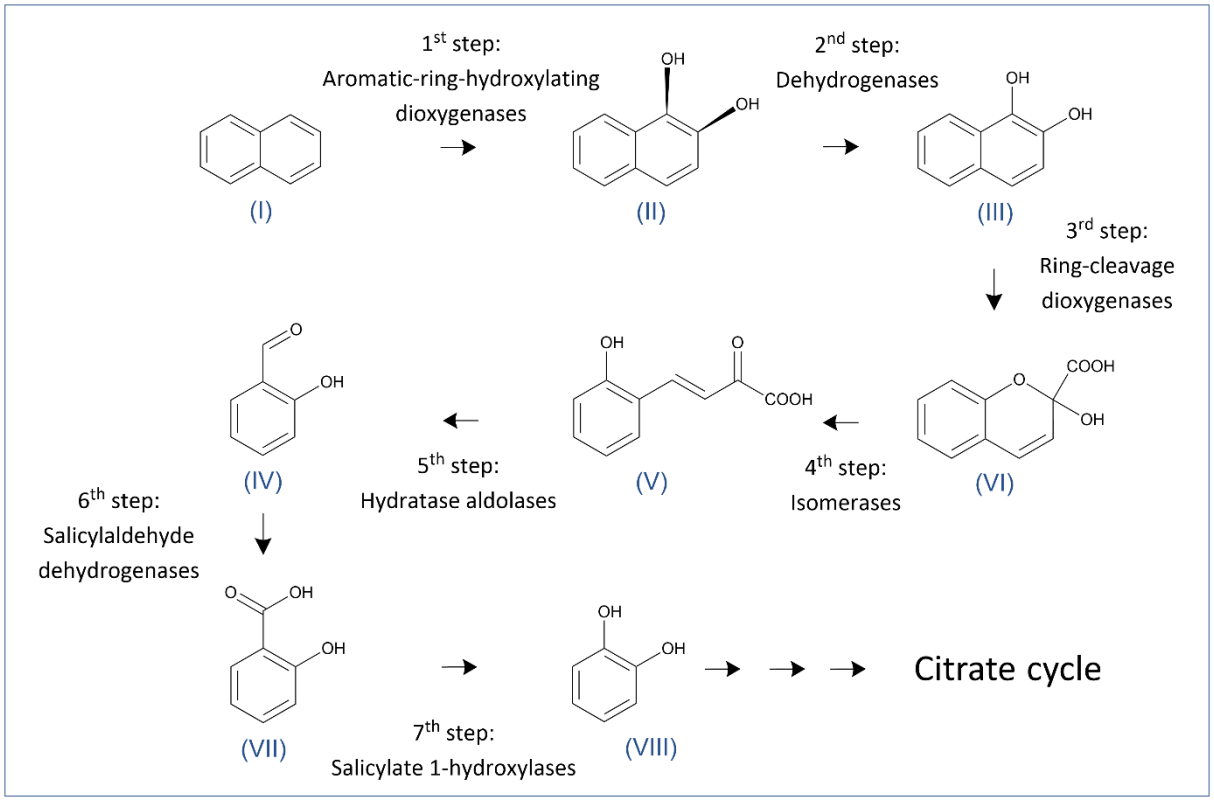

Fig. S1: Enzymes (labelled in the figure) and molecules involved in the biochemical naphthalene-degradation pathway in *Cycloclasticus spp.* (Wang *et al.*, 2018). (I) naphthalene, (II) *cis*-1,2-naphthalene-dihydrodiol, (III) 1,2-dihydroxynaphthalene, (IV) 2-hydroxy-2H-chromene-2-carboxylic acid, (V) *trans*-o-hydroxybenzylidene pyruvate, (VI) salicylaldehyde, (VII) salicylic acid, and (VIII) catechol.

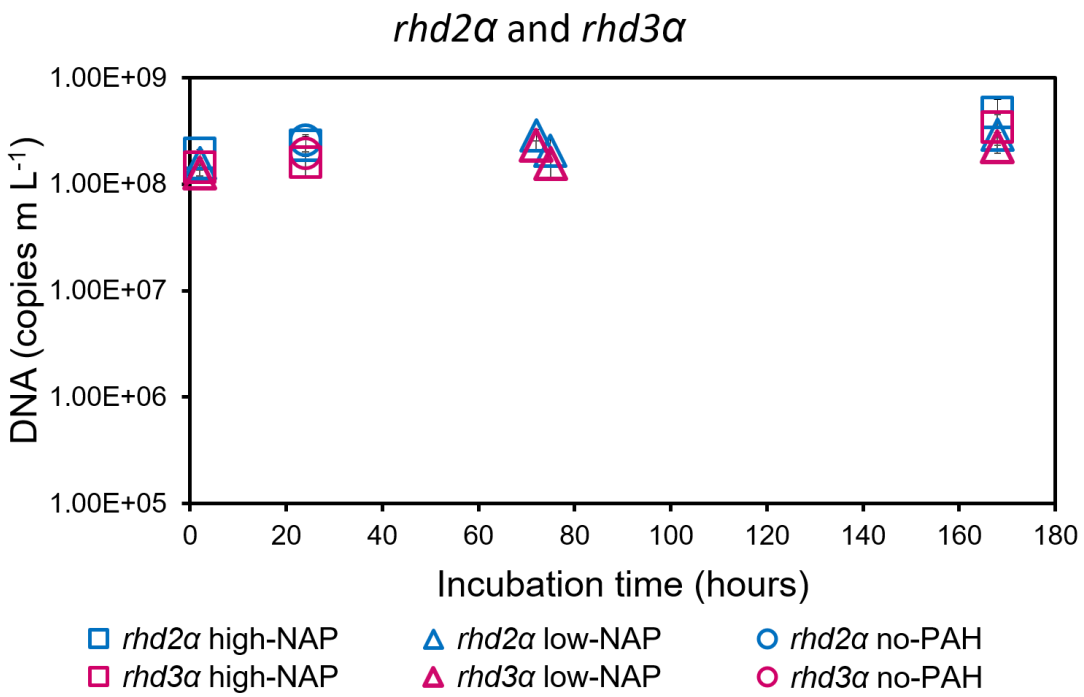

Fig. S2: Cell number estimates from DNA of two functional marker genes (*rhd2α* and *rhd3α* coloured blue and pink, respectively) in copies mL^{-1} quantified by qPCR in samples, which were selected for RNA sequencing. Incubation conditions were naphthalene at 100 mg L^{-1} and 30 mg L^{-1} (squares and triangles, respectively) as well as a PAH-free control, receiving pyruvate as carbon equivalent (circles). Error bars represent standard deviation between measured biological triplicates and are smaller than the markers.

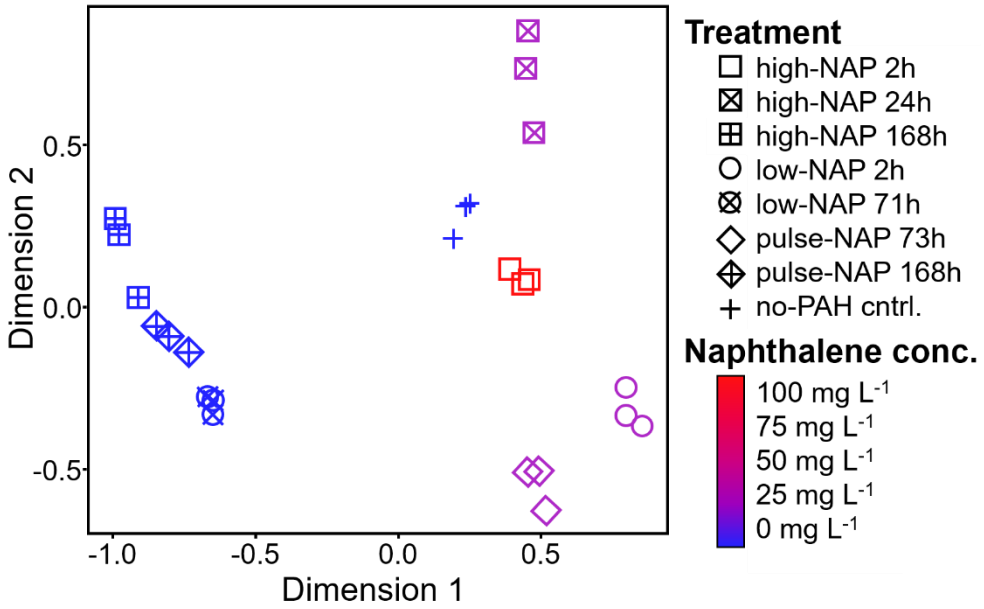

Fig. S3: Multidimensional scaling (MDS) plot of distances between gene expression profiles (all replicates displayed). Samples with similar gene expression are close to each other in the plot while samples that are dissimilar are further away from each other. Shapes indicate different treatments – high-NAP (squares), low-NAP (circles), pulse-NAP (diamonds) and no-PAH pyruvate control (cross). Color indicates naphthalene concentration high to low (red to blue).

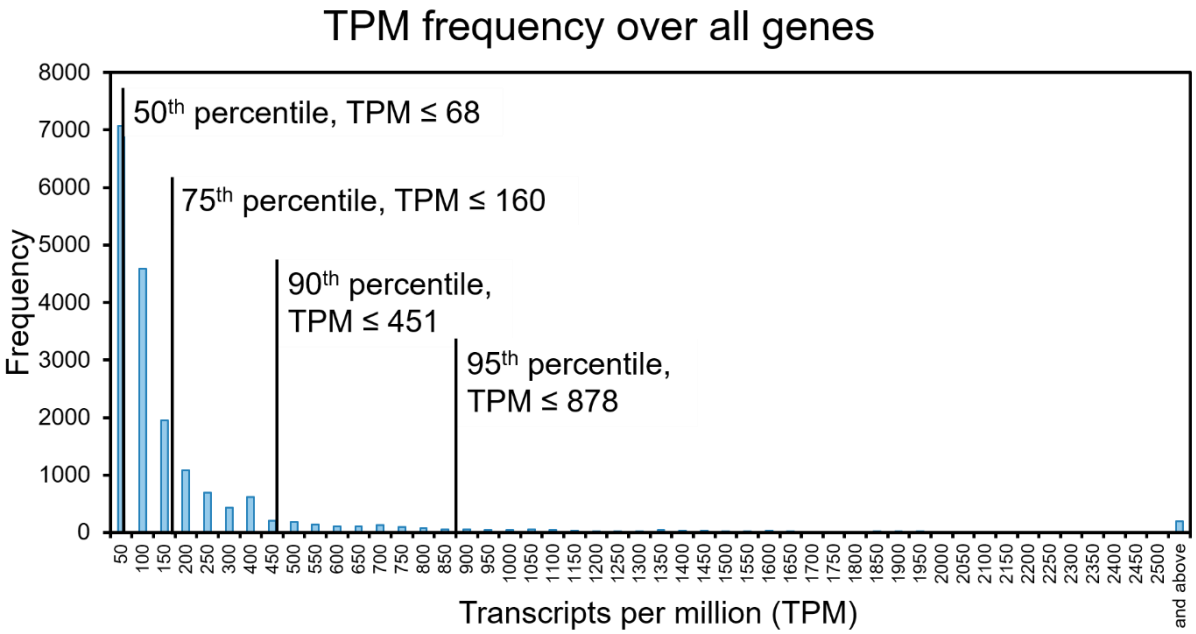

Fig. S4: Frequency of Transcripts per million (TPM) values of all genes over all treatments - 51 bins, 0 to 2500 in 50er bins. Marked 50th, 75th, 90th, and 95th percentiles were chosen to categorise expression levels, e.g. in Fig. 3,4, and 5.

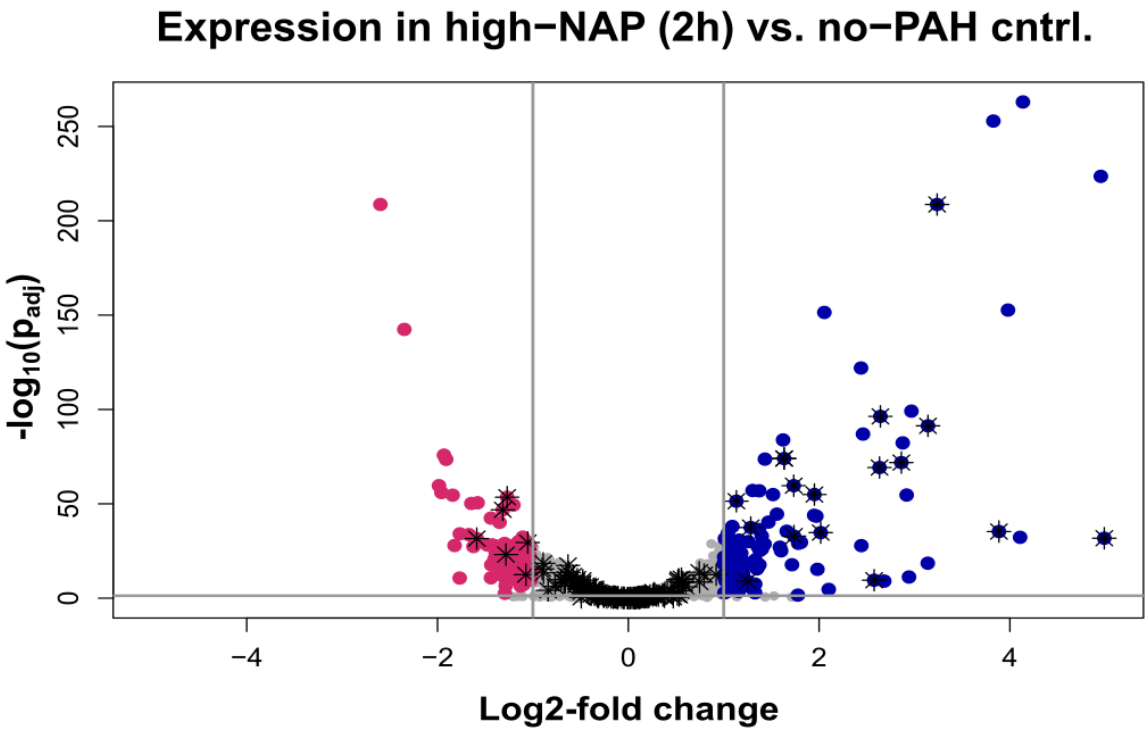

Fig. S5: Statistical significance ($-\log_{10}(p_{adj})$) versus the magnitude of change (log2-fold change) of – high-NAP 2h versus the no-PAH pyruvate control (24 h). Means per biological triplicates are displayed, significance ($p_{adj} \leq 0.05$) and change ($-1 < \log2\text{-fold change} > 1$) are indicated (pink and blue for down- and upregulation, respectively). 154 genes related to PAH-degradation curated from the literature are highlighted as black stars, also listed in Table S3, sheet A.

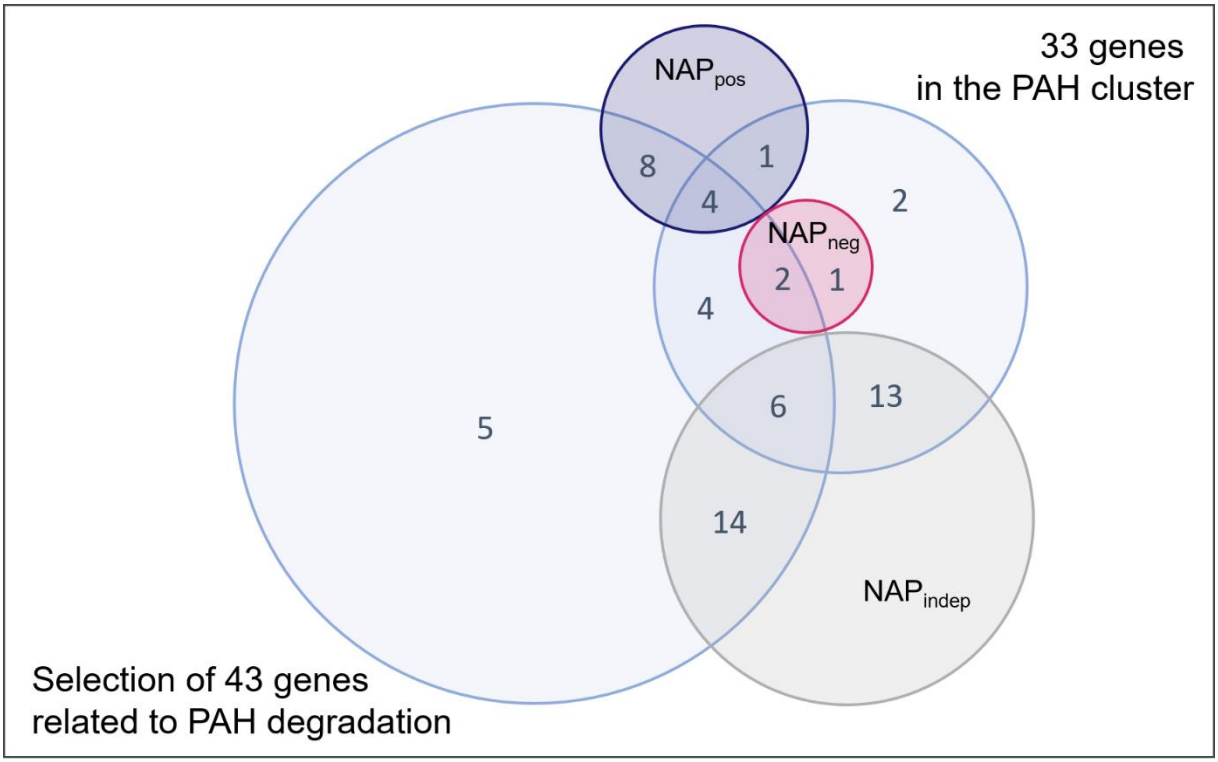

Fig. S6: Venn diagram illustrating intersection between subsets of investigated genes. The group of 43 genes (big light blue circle) was selected from the overall database containing 154 genes related to PAH degradation if TPM was within the 90th percentile in at least one naphthalene-containing treatment. The second main group represents the genes from a gene cluster previously associated with PAH degradation (Kasai *et al.*, 2003, Wang *et al.*, 2018). Small circles represent the categories with which the genes were associated based on their transcription in presence of naphthalene (see Table S4), NAP$_{pos}$ (dark blue), NAP$_{neg}$ (pink), and NAP$_{indep}$ (grey). The remaining genes – 5 for the selection, 2 for the genes from the cluster, and 4 from both – fell into the "no pattern" category (not represented by a separate circle).

TABLE S1 Primer characteristics for functional marker genes *rhd2α* and *rhd3α*.

Target gene	Primer name	Primer sequence 5' -> 3'	T_m [°C]	GC content [%]	Hairpin temp. [°C]	Primer-dimer temp. [°C]	qPCR product
rhd2α[1,2]	rhd2α1126F	ACA CGA AGA GGA AAG CTG CA	59.9	50	x	1	199 bp
	rhd2α1305R	TTT TCT TGC CTG CAT AGC GC	59.8	50	42.9	x	
rhd3α[2,3]	rhd3α 669FD	GGG TGG ACT AGC TGG AA	54.8	59	x	3.2	120 bp
	rhd3α781RD	TTC GCA TGA ATA GCG ATG G	55.9	47	59.1	11.2	

[1]Primer design from our previous study (Vogel *et al.*, 2023)
[2]Gene information (Wang *et al.*, 2018)
[3]Gene information (*rhd3α* is referred to as *phnA1*) and primer design from (Dionisi *et al.*, 2011)

TABLE S2 qPCR protocols for functional marker genes *rhd2α* and *rhd3α*. Volumes (10 µl in total) are given per reaction well.

	***rhd2α*[1,2]**	***rhd3α*[2,3]**
SYBER green	5 µL	5 µL
Primer F (0.5 µM)	1 µL	1 µL
Primer R (0.5 µM)	1 µL	1 µ
H_2O	2 µL	2 µL
Nucleotide tmpl.	1 µL	1 µL
Step 1	95°C, 5 min	95°C, 5 min
	1 cycle	*1 cycle*
Step 2 to 4	95°C, 20s	95°C, 20s
	62°C, 20s	62°C, 20s
		72°C, 20s
	40 cycles	*35 cycles*
Step 5	95°C, 1 min	95°C, 1 min
	1 cycle	*1 cycle*
Step 6	62°C, 30s	62°C, 30s
	1 cycle	*1 cycle*
Step 7	62°C – 95°C, 5s steps	62°C – 95°C, 5s steps
	melting curve	*melting curve*

[1]Primer design from our previous study (Vogel *et al.*, 2023)
[2]Gene information (Wang *et al.*, 2018)
[3]Gene information (*rhd3α* is referred to as *phnA1*) and primer design from (Dionisi *et al.*, 2011)

TABLE S3: Curated database of investigated genes related to PAH-degradation pathway in *Cycloclasticus pugetii* PS-1 with notes, references and expression data (mean TPM per biological triplicate) for each treatment. Sheet A) full set of 154 investigated genes, compiled using the annotations of the NCBI database (August 2022) (Schoch *et al.*, 2020), the KEGG database (August 2022) (Kanehisa & Goto, 2000, Kanehisa *et al.*, 2016, Kanehisa *et al.*, 2016), and published literature (Wang *et al.*, 1996, Kasai *et al.*, 2003, Wang *et al.*, 2018, Liang *et al.*, 2019, Wang *et al.*, 2021, Bagi *et al.*, 2022). Sheet B) genes selected if transcribed in naphthalene-containing treatments (mean TPM in at least one of the naphthalene-containing treatments within the 90th percentile, mean TPM ≥ 451) and sheet C) genes that are part of the previously investigated PAH-cluster (Kasai *et al.*, 2003, Wang *et al.*, 2018). **This table is available in an additional Microsoft Excel file.**

TABLE S4: Overview of the categorization of genes (categories NAP_{pos}, NAP_{neg}, NAP_{indep}, and no pattern) by transcriptional behaviour for each treatment. Transcription of the naphthalene-containing treatments is compared to the no-PAH controls and a gene is considered significantly up- (blue positive) or downregulated (pink negative) for $p_{adj} \leq 0.05$ and -1 < log2-fold change > 1, otherwise it is classified as PAH-independently expressed (grey indifferent). Genes are assigned to the NAP_{pos} and NAP_{neg} categories if the gene is up- or downregulated significantly in at least one of the treatments, even if some treatments show no change to the baseline.

	high-NAP 2 h	high-NAP 24 h	high-NAP 168 h	low-NAP 2 h	low-NAP 71 h	pulse-NAP 73 h	pulse-NAP 168 h	no-PAH cntrl.
NAP_{pos}	⊕	⊕	⊖	⊕	⊖	⊕	⊖	baseline
NAP_{neg}	⊖	⊖	⊕	⊖	⊕	⊖	⊕	baseline
NAP_{indep}	≈	≈	≈	≈	≈	≈	≈	baseline
no pattern	irregular expression	irregular expression	irregular expression	irregular expression	irregular expression	irregular expression	irregular expression	baseline

Table S5: Naphthalene concentration in abiotic and pyruvate controls, measured by GC-MS. Samples were measured in duplicates. Error represents standard deviation.

Treatment	Time = 0 h	Time = 24 h	Time = 73 h	Time = 168 h
high-NAP	106.1±0.20 mg L^{-1}	n.a.	n.a.	100.8±0.78mg L^{-1}
low-NAP	31.39 ± 0.81 mg L^{-1}	n.a.	n.a.	31.32±0.67mg L^{-1}
pulse-NAP	n.a.	n.a.	59.08±1.31 mg L^{-1}	59.94±1.42 mg L^{-1}
No-PAH cntrl.	n.a.	0.00±0.0 mg L^{-1}	n.a.	n.a.

Table S6: Genes that are part of the PAH gene cluster in *Cycloclasticus pugetii* PS-1. Highly expressed genes (mean TPM over all naphthalene containing treatments > 95th percentile) are marked in bold. Log2-fold change and p_{adj} are given for the comparison between treatments with 100 mg L^{-1} naphthalene input after 2 hours (high-NAP 2h) and no-PAH controls after 24 hours.

NCBI RefSeq assembly GCF_000384415.1/ Locus tag	**Putative function, *gene name(s)***	**log2-fold change (Nap/cntrl.)**	**p_{adj}**
CYCPU_RS0111430	(Hydroxy chromene-carboxylate) Isomerase, *phnD*[1,2]	-0.12	0.44
CYCPU_RS0111435	PAH dioxygenase component ferredoxin, *fer*, *phnA3*[1,2]	0.11	0.43
CYCPU_RS0111440	PAH dioxygenase component ferredoxin reductase, *pahA4*[1,2]	0.03	0.78
CYCPU_RS0111445	Hypothetical protein	0.27	0.01
CYCPU_RS0111450	Tryptophan synthase subunit β, *trpB*[1]	-0.12	0.36

CYCPU_RS0111455	Putative RHD β subunit, *orf7*[1]	1.97	1.74E-28
CYCPU_RS0111460	Ring cleavage dioxygenase, *rcd*, *phnC*[1,2]	-0.39	2.63E-3
CYCPU_RS0111465	RHD-3 β subunit, *rhd3β*, *phnA2*[1,2]	-0.52	1.72E-4
CYCPU_RS0111470	RHD-3 α subunit, *rhd3α*, *phnA1a*[1,2]	-0.47	2.30E-4
CYCPU_RS0111475	Aromatic dioxygenase large, *phnA1b*[1,2]	-0.04	0.82
CYCPU_RS0111480	Dihydrodiol dehydrogenas, *orf1*[1]	-0.33	1.29E-4
CYCPU_RS0111485	Hypothetical protein	-0.35	5.11E-3
CYCPU_RS0111490	RHD-PS1, Aromatic-ring-hydroxylating dioxygenase β, *rhdPS1β*	1.30	1.35E-13
CYCPU_RS0111495	RHD-PS1, Aromatic-ring-hydroxylating dioxygenase α, *rhdPS1α*	1.15	2.40E-22
CYCPU_RS0111500	DPR-2 regulator, *dpr2*	-0.06	0.56
CYCPU_RS0111505	RHD-9 α subunit, *rhd9α*	-0.15	0.45
CYCPU_RS0111510	RHD-9 β subunit, *rhd9β*	-0.65	1.16E-05
CYCPU_RS0111515	Short-chain dehydrogenase/reductase	-0.53	3.09E-5
CYCPU_RS0111520	Alcohol dehydrogenase	-0.50	3.26E-6
CYCPU_RS0111525	Outer membrane lipoprotein-sorting protein	-0.62	1.77E-9
CYCPU_RS0111530	Hypothetical protein	-1.26	8.85E-24
CYCPU_RS0111535	Multidrug efflux transporter, permease	-0.57	1.52E-6
CYCPU_RS0111540	Glycosyl hydrolase, BNR repeat	0.03	0.85
CYCPU_RS0111545	Alcohol dehydrogenase/quinone oxidoreductase	-0.14	0.40
CYCPU_RS0111550	Metabolite transporter superfamily, permease[2]	-0.10	0.63
CYCPU_RS0111555	RHD-2 α subunit, *rhd2α*[2]	-0.47	2.69E-3
CYCPU_RS0111560	RHD-2 β subunit, *rhd2β*[2]	-0.46	4.84E-09
CYCPU_RS0111565	Dioxygenase electron transfer component/ Carboxylate dehydrogenase	-0.65	5.87E-7
CYCPU_RS0111570	Glutathione S-transferase	-0.23	0.17
CYCPU_RS0111575	Hypothetical protein	0.39	0.02

CYCPU_RS0111580	Transglutaminase-like protein	0.05	0.72
CYCPU_RS0111585	Nitroreductase	-0.10	0.39
CYCPU_RS0111590	Outer membrane transport protein	0.54	7.29E-15

[1]Genes also part of "*phnA*-cluster" (Kasai *et al.*, 2003)
[2]Genes potentially regulated by DPR-2 regulator (Wang *et al.*, 2021)

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