



AS WE SEE IT

Isolation and cultivation of planktonic freshwater microbes is essential for a comprehensive understanding of their ecology

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ABSTRACT: Representative model organisms form the basis on which biology is constructed, and pure cultures offer many opportunities for discovery. However, our view of the importance of axenic cultures changed dramatically at the turn of the last century upon realizing that the majority of environmentally relevant microbes still remains uncultured. The sequencing revolution has led us to a point where we can identify the microbial world in which we live, but many questions remain regarding the autecology of planktonic microbes and their interactions with their environment. Thus, it is essential to isolate and cultivate the key microbial players to gain a deeper insight into their ecology. If the past is a guide, the way forward in confronting the so-called 'great plate count anomaly' is the use of more subtle and refined approaches to culturing, using a number of methods and processes that are now becoming available. The vast amount of information accumulated from genome sequencing alone has yet to result in the isolation of the most important and abundant microbes of aquatic systems. We highlight the merits of pure cultures and discuss the critical need to integrate information from a variety of different sources to isolate planktonic microbes. We also describe how to culture bacteria of interest with a full cycle isolation approach based on targeted enrichment and illustrate the benefits of pure cultures with 2 examples of isolated representatives of freshwater *Betaproteobacteria*.

KEY WORDS: Autecology · Axenic cultures · Dilution to extinction · Ecophysiology · Genomics · Isolation of freshwater microbes · Targeted enrichment

INTRODUCTION

Recent advances in sequencing and improved single-cell and microscopic imaging techniques suggest that the future of aquatic microbial ecology is largely based on (meta-)omics and *in situ* single-cell approaches (Stepanauskas 2012, Temperton & Giovannoni 2012, Blainey 2013, Son et al. 2015). Environmental meta-omics are undoubtedly important to gain insight into thus far unknown processes and functions of aquatic microbes (Rinke et al. 2013, Vila-Costa et al. 2013, Ghylis et al. 2014), and single-cell

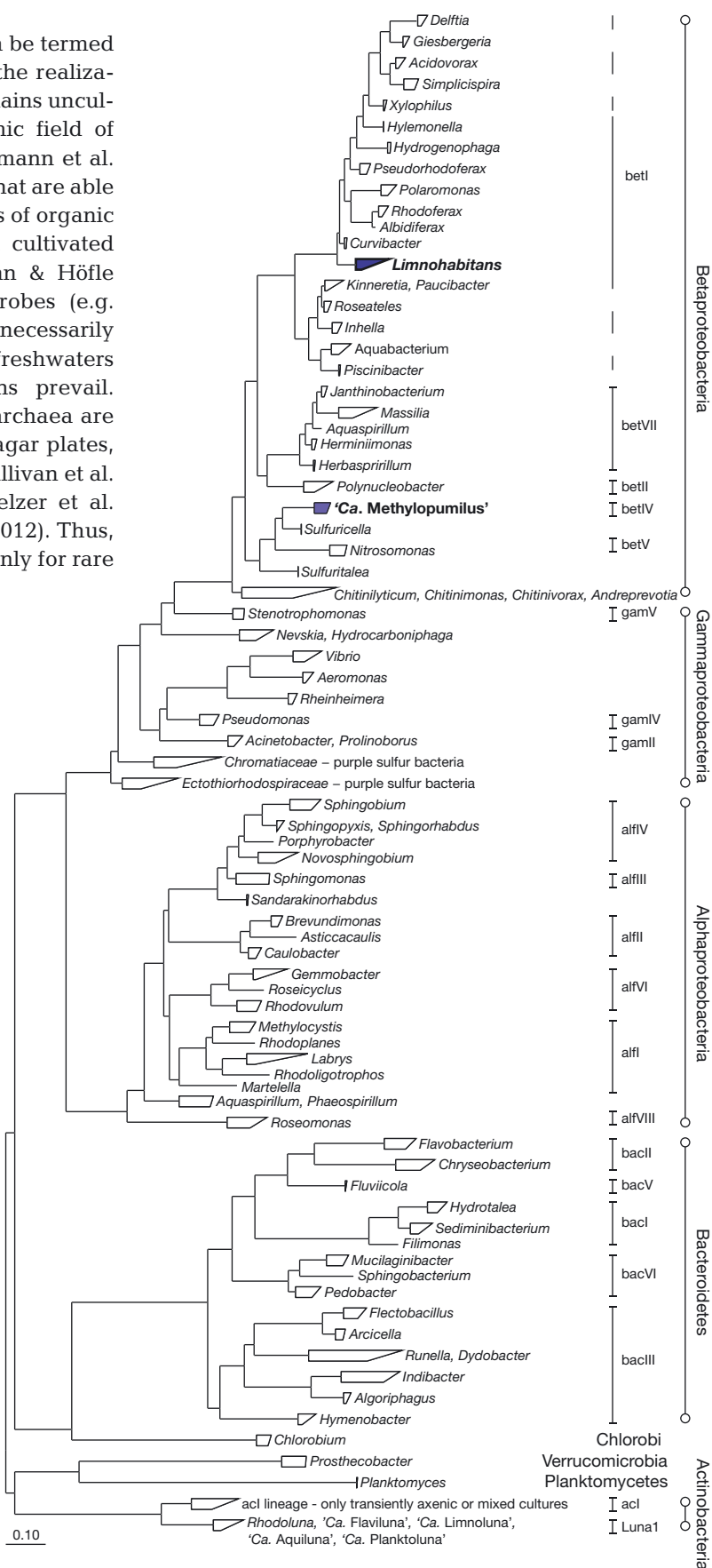
techniques allow for a precise quantification and/or functional allocation of individual cells or populations (Amann & Fuchs 2008, Musat et al. 2008, Stocker & Seymour 2012, Salcher et al. 2013). The main advantages of these methods are their *in situ* characteristics, i.e. the natural environment is not or only slightly disturbed during sampling; moreover, they do not rely on cultures and can be combined with experimental approaches.

However, it is still essential to isolate and cultivate the key players of aquatic systems to get 'a holistic picture' of their ecology (Giovannoni & Stingl 2007).

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At the turn of the last century, at what can be termed as the nascent time for the ‘-omics’ age, the realization that >99% of the microbial world remains uncultured came as a surprise in the dynamic field of microbiology (Staley & Konopka 1985, Amann et al. 1995). Some decades ago, only microbes that are able to grow in media with high concentrations of organic nutrients and carbon sources had been cultivated (Jannasch 1958, Allen et al. 1983, Hahn & Höfle 1998). However, such copiotrophic microbes (e.g. *Vibrio* spp., *Pseudomonas* spp.) do not necessarily represent the natural community in freshwaters where oligo- to mesotrophic conditions prevail. Moreover, as most aquatic bacteria and archaea are planktonic, they do not readily grow on agar plates, although there are some exceptions (O’Sullivan et al. 2004, Cousin et al. 2008, Hutalle-Schmelzer et al. 2010, Jogler et al. 2011, Watanabe et al. 2012). Thus, these cultivation approaches selected mainly for rare copiotrophic taxa, a phenomenon called ‘the great plate count anomaly’ (Staley & Konopka 1985, Amann et al. 1995). Further improvements in cultivation techniques, however, have led to the isolation of environmentally abundant oligo- to mesotrophic microbes (Fig. 1) of freshwater (Bruns et al. 2003, Hahn 2003, Hahn et al. 2003, Gich et al. 2005) and marine systems (Schut et al. 1997, Rappé et al. 2002, Könneke et al. 2005). As solid media proved to be inefficient for the isolation of obligate planktonic microbes, most cultivation efforts were made in liquid media and a dilution of the inoculum to 0.5–5 cells sample⁻¹ to obtain monoclonal cultures (dilution to extinction, serial dilution; Cannon & Giovannoni 2002, Bruns et al. 2003, Selje et al. 2005, Stingl et al. 2007). The main improvements for a successful isolation of microbes concerned the culti-

Fig. 1. Maximum likelihood tree of 16S rRNA genes of selected heterotrophic bacteria that were isolated from freshwaters and their affiliation to typical freshwater lineages proposed by Newton et al. (2011). The genera *Limnohabitans* and *Ca. Methylophilus* (highlighted in **bold**) are described in more detail in the main text. The scale bar at the bottom represents 10% sequence divergence



vation media that were either synthetic and adapted to natural oligotrophic conditions or sterilized lake water amended with only low amounts of nutrients, carbon sources, and vitamins (Bruns et al. 2003, Page et al. 2004). Another isolation strategy for very small microbes is the so-called filtration-acclimatization method invented by Hahn et al. (2003), where water samples are filtered through 0.2 μm membranes to remove larger organisms and the remaining ultramicrobacteria are adapted to higher concentrations of complex media in a stepwise manner. This method proved to be effective for the isolation of numerous freshwater strains affiliated with *Polynucleobacter* spp. and the Luna lineage of *Actinobacteria* (Hahn 2003, Hahn et al. 2003). Likewise, a selective enrichment of the target organisms prior to isolation can significantly increase the cultivation success. Such an enrichment of *Sphingomonadaceae* (*Alphaproteobacteria*) was achieved by the addition of growth inducers to minimal medium (Gich et al. 2005, Jogler et al. 2011) or by the addition of humic matter or phenol to sterile lake water (Hutalle-Schmelzer et al. 2010). The latter attempt also enabled enrichment and subsequent isolation of a wide range of different microbes from humic lakes (Hutalle-Schmelzer et al. 2010). Microbes which grow mainly on algal-derived organic matter (e.g. *Flavobacteriaceae*, *Comamonadaceae*) can be enriched upon addition of algal extracts or exudates to the cultivation medium or might be isolated at times of algal blooms (Zeder et al. 2009, Hahnke et al. 2015, Salcher et al. 2016). Finally, fast-growing microbes like *Limnohabitans* spp. or *Flavobacterium* spp. can also be enriched in predator-free dilution cultures (Kasalický et al. 2013, Neuenschwander et al. 2015). One example of a successful isolation of such fast-growing bacteria (*Limnohabitans* spp.) is described in more detail below.

POSSIBLE REASONS WHY SOME OBLIGATE PLANKTONIC MICROBES STILL RESIST CULTIVATION

A large number of prokaryotes isolated from freshwaters have been validly described (Fig. 1). However, the majority of these taxa have been isolated on agar plates or in rich media and therefore represent typical copiotrophs or 'tychoplankton,' i.e. they are transient or not very abundant members of the plankton, as signature sequences of these microbes have rarely been recovered from environmental samples (Newton et al. 2011). Notably, a

valid description of a novel species requires a deposition of the type strain in 2 public culture collections to make it available to other scientists (Kämpfer et al. 2003), and a prerequisite for the deposition is a successful cultivation in synthetic media and/or on agar plates. Obligate planktonic prokaryotes that are hard to cultivate rarely fall into this category. For example, the most abundant marine microbes—the SAR11 clade—are still not validly described ('*Ca. Pelagibacter ubique*') and it took more than 10 yr from the isolation of the first SAR11 strains to a successful cultivation in defined synthetic medium (Rappé et al. 2002, Carini et al. 2013). Only recently, a number of environmentally relevant planktonic freshwater taxa have been brought to culture (Fig. 1), e.g. *Limnohabitans* spp. (Hahn et al. 2010a,b, Kasalický et al. 2010, 2013), *Polynucleobacter* spp. (Hahn et al. 2009, 2010c, 2011, 2012), '*Ca. Methylopusillus* spp.' (Salcher et al. 2015), *Sphingomonas* spp. (Hutalle-Schmelzer et al. 2010, Jogler et al. 2011, Salka et al. 2014), *Rhodoluna* spp. (Hahn et al. 2014), *Fluviicola* spp. (O'Sullivan et al. 2005), and *Flavobacterium* spp. (Cousin et al. 2008, Ali et al. 2009, Sack et al. 2011, Lee et al. 2012).

The most abundant planktonic freshwater microbes, i.e. *Actinobacteria* of the acI-lineage and *Alphaproteobacteria* of the LD12 lineage (the freshwater sister group of SAR11), however, still resist axenic cultivation. Both lineages are of very small cell size (ultramicrobacteria), have streamlined genomes, and follow a typical oligotrophic lifestyle with adaptations to very low nutrient and carbon concentrations (Salcher et al. 2011b, Zaremba-Niedzwiedzka et al. 2013, Ghylis et al. 2014). Streamlined oligotrophs are typically non-motile, seem to be only slowly growing, and are characterized by poor metabolic plasticity, i.e. they are unable to acclimate to resource-rich conditions. However, they are extremely competitive in nutrient-poor conditions of natural aquatic habitats and seem to at least partially escape predation by protists (Yooseph et al. 2010). These ecological features together with specific nutritional requirements and potential auxotrophies for unknown compounds may have hampered successful isolation thus far. For example, although members of the acI lineage have been repeatedly isolated or enriched via dilution to extinction (Gich et al. 2005, Selje et al. 2005, M. M. Salcher unpubl. data), the initially dense monocultures stopped growing when propagated to fresh sterile lake water medium, and all attempts to keep cultures alive have so far been unsuccessful

(M. M. Salcher unpubl. data). This hints at very specific adaptations to the environmental conditions at the time of isolation, such as the presence of vital unknown growth substrates. Nevertheless, biomass of these transiently cultivable microbes can be used for whole-genome sequencing, thus providing information about specific metabolic pathways that might help to refine potential cultivation media (M. M. Salcher unpubl. data). AcI *Actinobacteria* can be grown in co-cultures for several generations (Jezbera et al. 2009, Garcia et al. 2014), and 1 species has been so far described as *Candidatus* ('*Ca. Planktophila limnetica*,' Jezbera et al. 2009). It is thus very likely that acI *Actinobacteria* live in close contact and metabolic interconnectedness with co-occurring microbes, i.e. they depend on metabolites provided by others (Garcia et al. 2015, Garcia 2016). *Actinobacteria* of the Luna lineage on the other hand can be grown axenically in rich complex medium, although they are also of very small size and have reduced genomes (Hahn et al. 2003, 2014, Hahn 2009).

BENEFITS OF PURE CULTURES

High-quality reference genomes

Whole-genome sequencing and assembly is much easier and cheaper from monocultures than from mixed assemblages or single amplified cells (SAGs), and unbiased high-quality reference genomes can only be produced from axenic cultures. Genomes assembled from metagenomic reads (MAGs) are always a composite of closely related taxa, as it is simply impossible to reconstruct genomes of individual strains in a mix of co-existing genotypes (Temperton & Giovannoni 2012). SAGs, on the other hand, are intrinsically incomplete because of difficulties in the flow cytometric sorting, whole-genome amplification, and sequencing of DNA from single cells (Woyke et al. 2011, Clingenpeel et al. 2014). Therefore, it cannot be assessed whether particular genes or pathways are indeed absent or incomplete in SAGs and whether particular genomic traits are specific for single genotypes in MAGs.

Reference for meta-omics and detection of different genotypes in nature

Closed high-quality genomes from axenic cultures can serve as references for a variety of meta-omics approaches and for the design of specific primers and probes (Fig. 2). Indeed, fragment recruitment of metagenomic reads to full genome sequences has been repeatedly used to identify hypervariable regions or metagenomic islands hinting at a high level of microdiversification within closely related strains in the environment (Rodriguez-Valera et al. 2009, Cordero & Polz 2014, Thrash et al. 2014). Genome sequences can also be retrieved from metatranscriptomes and -proteomes (Fig. 2); a mapping of reads gives vital information on transcribed genes under different environmental conditions and can shed light on their role in the environment. For example, this was demonstrated for the abundant marine bacterium *Planktomarina temperata* during a phytoplankton bloom (Voget et al. 2015). The design and testing of strain- or lineage-specific probes for fluorescence *in situ* hybridization (FISH) or specific primers

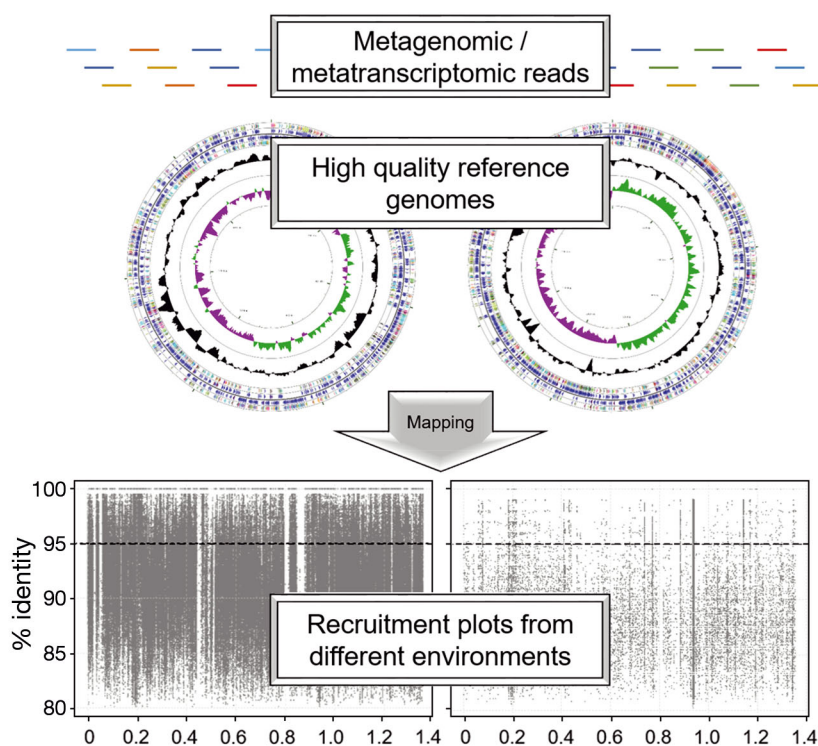


Fig. 2. Workflow for the usage of high-quality genomes from isolates as a reference for meta-omics. Fragment recruitment of metagenomic (left) and metatranscriptomic (right) reads to full genome sequences provides valuable information on the numerical relevance and potential microdiversification of different genotypes

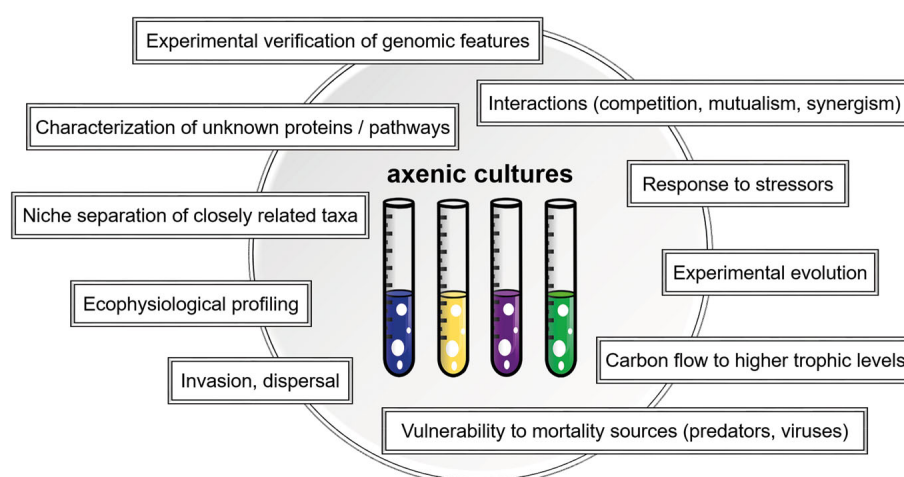


Fig. 3. Ideas for possible experiments with axenic cultures of freshwater microbes

for qPCR (Ramachandran & Walsh 2015) and related applications (e.g. reverse line blot hybridization, RLBH: Jezbera et al. 2011; terminal restriction fragment length polymorphism: Paver et al. 2015) is straightforward from genomic data from cultures. Such primers or probes facilitate investigations of the abundance of cultured taxa in different ecosystems and can be used to test for microdiversification, habitat preferences, and seasonal dynamics of closely related strains.

Autecology studies

Metabolic and ecophysiological traits of different taxa can be easily examined with cultures, e.g. specific growth rates, substrate and nutrient requirements, temperature and salinity optima, etc. (e.g. Hahn & Pöckl 2005, Ali et al. 2009, Kasalický et al. 2013). Although the genomic potential of uncultivated microbes can also be assessed with metagenomics and SAGs, the mere presence or—in the case of (meta-)transcriptomics—expression of functional genes should not be over-interpreted, as it sometimes gives only limited information about specific ecophysiological functions in the target microbes. Single-cell methods can provide insights into the *in situ* substrate acquisition of microorganisms; however, they are usually restricted to a few substrates and are very laborious (Wagner et al. 2006, Salcher et al. 2013). Moreover, closely related strains of the same species or genus might differ in ecophysiological properties, making meta-omic and FISH-based analyses complicated due to the limited taxonomic resolution of these methods. Thus, autecological

studies of cultured microbes provide much more detail and are also easier and faster to do. Functional postgenomic analyses (transcriptomics, proteomics, or metabolomics) of genome-sequenced strains growing under different conditions can help to identify their metabolic capacities (Zech et al. 2009, Smith et al. 2013)

Experimental approaches and hypothesis testing

Numerous laboratory or *in situ* experiments can be set up with axenic cultures (Fig. 3). Ecophysiological profiling of different strains can be combined with ‘-omics’ (e.g. transcriptomics, proteomics) and thus give valuable insights in the regulation of central metabolism under different cultivation regimes (Sowell et al. 2008, Zech et al. 2009, Steindler et al. 2011, Smith et al. 2013). One big challenge in microbial genomics is that a large fraction of genes cannot be assigned to a specific function (ca. 20–30%). Such genes or proteins of unknown function can be either characterized by detecting homologues in closely related organisms or by experimental evidence from cultures (e.g. via gene overexpression), although this is very laborious and time consuming. However, only the latter can ultimately identify unknown or unexpected metabolic pathways. For example, the genome of ‘*Ca. Pelagibacter ubique*’ encodes an unusual glycine-riboswitch that is essential for the central carbon metabolism (Tripp et al. 2009). Several ‘*Ca. Pelagibacter ubique*’ genomes have been screened for other unexpected metabolic pathways, and accompanying experimental work uncovered that these microbes are ‘methylovores,’ i.e. they can

oxidize 1-carbon (C_1) compounds for energy generation (Sun et al. 2011). Such genome-assisted studies of laboratory cultures can also test for specific hypotheses that were generated during genome analyses or field investigations, e.g. adaptations to different substrates, temperatures, or salinity regimes (e.g. Hahn & Pöckl 2005, Salcher et al. 2015). Finally, interactions between different organisms like inter- and intraspecific competition for limiting nutrients and substrates, commensalistic, mutualistic, or synergistic effects (e.g. Jagmann et al. 2010, González-Torres et al. 2015), as well as the vulnerability to different mortality sources (grazing by protists, viral lysis) and potential microbial defense mechanisms can be addressed with isolates (Fig. 3). Predators or phages can be identified and isolated by using cultures as prey or hosts (Šimek et al. 2013, Zhao et al. 2013) and allow a detailed analysis of the fate of distinct taxa and their role in the carbon transfer to higher trophic levels. Other experimental approaches might target the response of different taxa to stressors (e.g. salinity, UV irradiation, antibiotics), invasion, dispersal, and adaptations to new environments (Hornák & Corno 2012, Hall & Corno 2014). To sum up, the list of potential experiments that can be set up with cultures is almost endless. Examples of 2 bacterial taxa (*Limnohabitans* spp. and '*Ca. Methylo-pumilus* spp.') that were used in several experimental approaches are described in detail below.

Establishment of new taxa

A valid description of new taxa is only possible if strains can be easily cultivated in monocultures in synthetic media and/or on agar plates and is thus hardly practicable for obligate planktonic oligotrophs (see above). However, a proposal as *Candidatus*, i.e. taxa that cannot be described in sufficient detail to warrant establishment of a novel taxon, circumvents the strict rules of the bacteriological code and might be applicable for microbes that are hard to isolate and grow (Murray & Stackebrandt 1995). Both options, the proposal of a new taxon or a *Candidatus*, enable a better description and formal naming of environmental relevant genera and species and should include a list of ecological, phenotypic, and genotypic traits. Consistent naming of closely related microbes might also help to improve the quality of public sequence databases that include so far mainly 'uncultivated bacteria' and to make straightforward cross-study comparisons (Newton et al. 2011).

Establishment of new model systems for different types of freshwater microbes

There is a need for more and better model organisms from freshwaters, i.e. all broadly defined ecotypes of freshwater microbes should have at least 1 cultivated representative serving for experimental studies and genetic engineering. While specialized microbes that play vital roles in the S, N, CH_4 , Fe, or H-cycle (e.g. sulfur oxidizers and reducers, nitrifying and denitrifying bacteria) have many cultivated taxa, model systems for the numerically dominant planktonic microbes that degrade different types of dissolved organic carbon (aerobic chemo-organoheterotrophs) are still rare. Thus, more planktonic microbes need to be isolated to get additional model systems, as not only the pool of dissolved organic matter but also their potentially specialized degraders are very diverse.

HOW TO ISOLATE PLANKTONIC MICROBES? THE 'FULL CYCLE ISOLATION APPROACH' BASED ON TARGETED ENRICHMENT

Analogous to the 'full cycle rRNA approach' (Amann et al. 1995) and the 'full cycle metagenomics approach' (Bodrossy 2015), we propose a 'full cycle isolation approach' that builds on ecological data gained from exploratory studies (Fig. 4). Genome sequences from MAGs or SAGs are a backbone for this approach, but data might also derive from temporal or spatial quantification of microbes in different environments, from *in situ* experiments, *in situ* eco-physiology studies (e.g. MAR-FISH, metatranscriptomics, stable isotope probing; Wagner et al. 2006, Wagner 2009, Franzosa et al. 2015), or any other method that gives specific hints on the ecology of the target microbes. Basically every published article addressing the organism of interest adds another piece of information to this composite ecological picture and might help to identify factors that stimulate its growth. These ecological features can be used for targeted enrichment of the preferred organisms in manifold ways: raw water samples can be amended with specific substrates (e.g. algal extracts for *Flavobacteriaceae*, humic matter for *Sphingomonadaceae*, methanol for *Methylophilaceae*; see 'Introduction' for details and more examples), or a synthetic cultivation medium can be designed based on reconstructed metabolic pathways. Other means to enrich the target microbes in natural samples include food web manipulations (e.g. the addition or removal of bac-

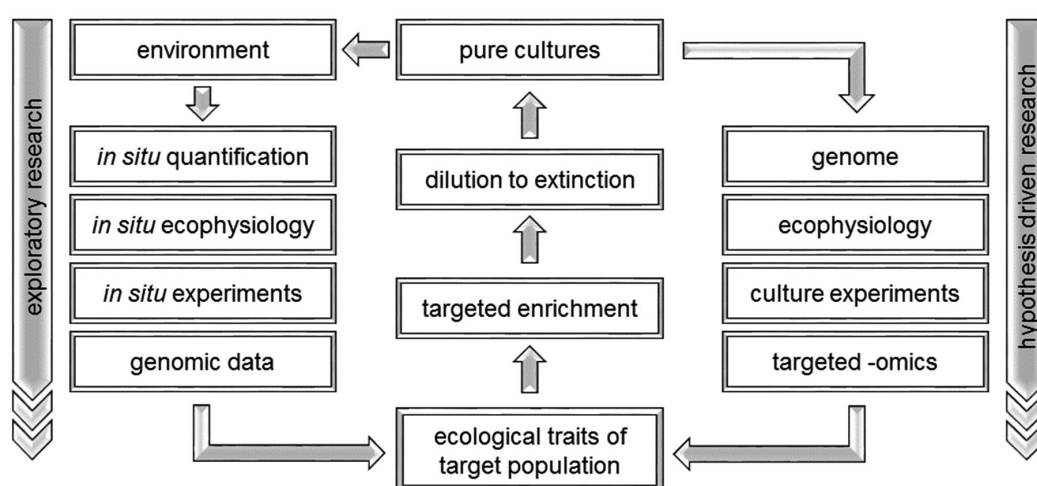


Fig. 4. Summary of the 'full cycle isolation approach' for a targeted isolation of microbes

terivorous predators), physical exclusion of bigger organisms (filtration through 0.2 or 0.45 μm membranes), or flow cytometric sorting of distinct populations. One should keep in mind that targeted enrichment is a trial-and-error approach that might sometimes lead to unexpected results. However, successful enrichments can subsequently be purified to clonal cultures by dilution to extinction. In addition, the design of new imaginative growth media can also be based on the interpretation of ecological or genomic data (Carini et al. 2013). To close the circle, pure cultures can be used to find exactly these genotypes in nature, to do hypothesis-driven experiments, and to study their geno- and phenotypic traits in depth to get more insights into their ecophysiology (Fig. 4).

TWO EXAMPLES OF A SUCCESSFUL APPLICATION OF THE 'FULL CYCLE ISOLATION APPROACH'

Ideally, single-cell methods, *in situ* quantification, phenotypic characterization, and '-omic' approaches should be combined to study the most abundant freshwater microbes. The availability of cultures has accelerated development in several research lines where environmental meta-omics and single-cell methods can hardly achieve sufficient species-specific resolution. The examples below describe a successful application of the 'full cycle isolation approach' for 2 ubiquitous betaproteobacterial genera. We briefly summarize the timeline from first discoveries of 16S rDNA sequences in clone libraries to a successful targeted isolation based on a mosaic of ecological features that evolved from exploratory

studies. Moreover, we report examples of genomic traits, specific substrate utilization, distribution in different environments, and experimental approaches that address questions related to niche separation, vulnerability to different mortality sources, and their role in carbon flow to higher trophic levels.

Limnohabitans spp.

Environmental 16S rDNA sequences gained from several freshwater ecosystems indicated a ubiquitous distribution of the beta I or '*Rhodferax* sp. BAL47' lineage of *Betaproteobacteria* (Glöckner et al. 2000, Zwart et al. 2002). These 16S rDNA sequences allowed the design of a specific probe for FISH (R-BT065, Šimek et al. 2001) that was intensely used. For instance, these bacteria were present in high numbers in a wide range of freshwater habitats, and their abundances were positively correlated to higher pH and concentrations of low-molecular-weight dissolved organic carbon (Warnecke et al. 2005, Šimek et al. 2010b). Temporal monitoring in single habitats revealed a pronounced seasonality with maxima during phytoplankton blooms in spring (Selje et al. 2005, Grossart et al. 2008, Šimek et al. 2008, 2014, Eckert et al. 2012, Salcher 2014) and/or summer (Salcher et al. 2008, Buck et al. 2009, Pérez & Sommaruga 2011). Moreover, the FISH probe was also used in various *in situ* experiments that resulted in first insights in the ecology of *Limnohabitans* spp. that is characterized by: (1) a rapid growth in response to environmental changes (Šimek et al. 2006, Neuenschwander et al. 2015), (2) a high substrate versatility (Salcher et al. 2013, Rofner et al. 2016a) and uptake rates (Horňák

et al. 2006, 2008, Salcher et al. 2008, Pérez et al. 2015, Rofner et al. 2016b), (3) a high level of vulnerability to predation by flagellates (Jezbera et al. 2005, 2006, Šimek et al. 2005, 2014), and (4) a strong link to phytoplankton-derived organic material as a key growth substrate (Pérez & Sommaruga 2006, 2007, Šimek et al. 2008, 2011, Horňák et al. 2012, Paver et al. 2013).

Based on these ecological features, numerous strains of the genus have been brought to culture by targeted isolation. As *Limnohabitans* spp. are among the fastest-growing microbes in bacterivore-free treatments (plankton samples filtered through 0.8 µm, Šimek et al. 2001, 2006) they were used to develop a modified filtration-acclimatization protocol (Hahn et al. 2010a,b, Kasalický et al. 2010, 2013): water samples were filtered through a 0.8 µm polycarbonate membrane and the filtrate was left for 12 h at 18°C, which resulted in an approximate doubling of the rapidly growing target group. The filtrate was subsequently diluted with inorganic basal medium and inoculated onto 24-well plates (~0.5 cells well⁻¹), and cultures were acclimatized to growth in rich medium by stepwise addition of increasing doses of NSY medium (Hahn et al. 2003). This protocol has facilitated the isolation of more than 45 strains of diverse sizes and morphologies and establishment of the genus *Limnohabitans* with 4 validly described species to date (Hahn et al. 2010a,b, Kasalický et al. 2010). Although several strains affiliated with *Limnohabitans* spp. were already isolated earlier, they were not further investigated and not validly described (Page et al. 2004, Gich et al. 2005, Selje et al. 2005).

The intergenic spacer between the 16S and 23S rRNA gene (ITS) was further used as a fine-scale marker and resulted in the delineation into 5 lineages (LimA, LimB, LimC, LimD, and LimE) and several sublineages within the most diversified lineage LimC (Kasalický et al. 2013). These lineages could also be discriminated by large differences in cell size (0.02–0.9 µm³ cell volume), morphology (coccoid, rods, curved, solenoid, ovoid), and substrate uptake (Kasalický et al. 2013). A profound microdiversification and habitat preference of the different lineages across a large set of freshwater systems was discovered with RLBH probes designed from ITS sequences (Jezbera et al. 2013). Likewise, whole-genome sequencing of 22 strains revealed a large diversity in genome size (2.6–5 Mbp) and genomic traits, e.g. several alternative ways of bacterial phototrophy and CO₂ fixation were discovered in some strains of this genus that was formerly assumed to be entirely

heterotrophic (Zeng et al. 2012, V. Kasalický unpubl.). One lineage related to *L. planktonicus* was repeatedly detected as symbionts of *Daphnia* sp.; thus, not all *Limnohabitans* spp. live exclusively planktonic (Freese & Schink 2011, Eckert & Pernthaler 2014, Peerakietkhajorn et al. 2016). The genus is also not only restricted to freshwaters, as some genotypes occurred in brackish environments (Alonso et al. 2009, Piwosz et al. 2013). *Limnohabitans* strains have repeatedly been used in experimental studies with simplified microbial communities to assess niche separation among coexisting strains through interactions with particular algal species and their exudates (Šimek et al. 2011), their vulnerability to predation by protists or viral infection, and interspecific interactions with other bacteria (Šimek et al. 2010a, Horňák & Corno 2012, Hall & Corno 2014, Salcher et al. 2016). Their role in the carbon flow to higher trophic levels has been studied in detail in experiments using protistan model organisms (Šimek et al. 2010a, Salcher et al. 2016) or natural heterotrophic flagellate communities from different lakes (Šimek et al. 2013, Grujić et al. 2015). All of these experiments proved that *Limnohabitans* spp. are of high food quality for protists and have a limited ability to form grazing-resistant morphologies. They thus play an important role in channeling carbon to higher trophic levels in aquatic food webs, although with striking strain-specific as well as season- and site-specific patterns. In summary, the genus *Limnohabitans* is very diverse, and individual strains differ dramatically in their ecology; however, they also have common features, i.e. fast growth and high importance in microbial food webs. Thus, *Limnohabitans* spp. represent perfect model organisms for diversified copiotrophs with high environmental relevance.

‘Ca. Methylopumilus spp.’

Similar to *Limnohabitans*, the beta IV or LD28 lineage of *Betaproteobacteria* was also first discovered from environmental 16S rDNA sequences (Zwart et al. 1998, 2002, Glöckner et al. 2000) and frequently recovered thereafter (Newton et al. 2011). The application of a general FISH-probe targeting the whole family (*Methylophilaceae*) resulted in high numbers of planktonic microbes in hypolimnetic samples, while they were rare in surface samples during summer (Salcher et al. 2008, Jezbera et al. 2012). The development of a specific probe (LD28-1017) proved this spatial distribution (Salcher et al. 2011a). Their

close phylogenetic relation to planktonic marine (Giovannoni et al. 2008) and freshwater sediment methylotrophs (Chistoserdova 2015) hinted at a methylotrophic lifestyle, i.e. a specialization to C_1 substrates. Indeed, MAR-FISH revealed no or only a low uptake of amino acids and sugars (Salcher et al. 2008, 2013).

The isolation of a first strain was reported early (Gich et al. 2005), but this strain was not further investigated. Dilution to extinction with sterile filtered and autoclaved lake water as medium and a 0.4 μm filtered inoculum resulted in the isolation of >120 strains, and the LD28 lineage as well as the closely related PRD01a011B lineage were described as *Candidatus* ('*Ca. Methylopumilus planktonicus*' and '*Ca. M. turicensis*;' Salcher et al. 2015). A recently modified targeted isolation approach with size fractionation and enrichment in artificial medium containing only methanol and methylamine as carbon sources resulted in a greatly enhanced cultivability of planktonic methylotrophs with >90 new isolates from different lakes (M. M. Salcher unpubl. data).

All strains affiliated with '*Ca. Methylopumilus planktonicus*' are of conspicuous small cell size (0.02–0.07 μm^3 , i.e. ultramicrobacteria) and display very slow growth ($\mu_{\text{max}} = 0.4 \text{ d}^{-1}$). Whole-genome sequencing of several strains revealed very small and streamlined genomes (1.3 Mbp) with a low GC content and a reduced number of genes encoding methylotrophic pathways compared to their relatives from freshwater sediments. Therefore, these microbes follow a typical oligotrophic lifestyle, similar to their marine sister lineage OM43 (Giovannoni et al. 2008). The genomes lacked genes for methylamine oxidation, thus these microbes seemed to be unable to utilize this C_1 compound. However, growth of one strain was enhanced upon addition of methylamine to sterile lake water, hinting at so far unknown pathways or genes (Salcher et al. 2015). Genomic sequences of the gene encoding methanol dehydrogenase (*xoxF*) from '*Ca. Methylopumilus* spp.' and the marine OM43 were used to search in numerous metagenomes and to develop specific qPCR primers that enabled a quantification of gene numbers and mRNA transcripts in different freshwater, estuarine, and marine habitats (Ramachandran & Walsh 2015). This gene had a widespread distribution in lakes, rivers, and coastal marine sites, and highest expression coincided with a phytoplankton bloom (Ramachandran & Walsh 2015). This is in accordance with seasonal monitoring of the abundances of '*Ca. Methylopumilus* spp.' in different lakes, where max-

ima occurred concomitantly with blooms of diatoms and/or cyanobacteria (Li et al. 2015, Salcher et al. 2015, Woodhouse et al. 2016), indicating that C_1 substrates supporting their growth were presumably released from primary producers. Seasonal monitoring also suggested an adaptation to cold water temperatures, a hypothesis that was verified by the observation that cultures reached higher densities at colder incubation temperatures (Salcher et al. 2015). In summary, '*Ca. Methylopumilus* spp.' are perfect model organisms for specialized oligotrophs with very reduced genomes, and their close phylogenetic relationship to planktonic marine and freshwater sediment microbes makes them ideal for evolutionary studies regarding genome streamlining, horizontal gene transfer, habitat transitions, and ecological specialization (Walsh et al. 2013, Ramachandran & Walsh 2015, Salcher et al. 2015, Jimenez-Infante et al. 2016).

CONCLUSIONS AND FUTURE PERSPECTIVES

We hope that our presented ideas and examples may inspire readers (1) to enrich and isolate planktonic freshwater microbes (Fig. 4), (2) to set up experiments with cultured strains (Fig. 3), and (3) to use fine-resolution '-omics' approaches in combination with classical ecophysiological profiling and *in situ* methods. Continuing efforts will hopefully result in the successful isolation of more freshwater microbes, especially of those that have thus far been elusive. The 'full cycle isolation approach' for targeted cultivation (Fig. 4) might be a future direction, as the increasingly available tools of (meta-)omics shed more light on the ecology of so far uncultivated taxa which might help to enrich and isolate the target microbes and to design specific media that support their growth.

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