Vol. 77: 79–85, 2016
doi: 10.3354/ame01789

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online August 12

Contribution to AME Special 6 'SAME 14: progress and perspectives in aquatic microbial ecology'

AS I SEE IT

Mixed cultures as model communities: hunting for ubiquitous microorganisms, their partners, and interactions

Sarahi L. Garcia*

Department of Ecology and Genetics, Limnology, Uppsala University, Norbyvägen 18 D, 75236 Uppsala, Sweden

ABSTRACT: Even though thousands of microbial strains have now been successfully cultivated and described, these only represent a small fraction of global microbial diversity. Moreover, many of the ubiquitous and abundant environmental microorganisms still defy axenic cultivation. Here, I present mixed cultures as a powerful tool to cultivate and study ubiquitous but hard-to-cultivate microorganisms. A mixed culture is a subsample from a complex natural community that contains 2 or more microbial strains. When cultivated together with their metabolic partners, these ubiquitous microorganisms can mutually satisfy metabolic dependencies just as they do in the environment. By reducing the complexity while keeping some diversity, mixed cultures can then be used as model communities. Furthermore, by combining the relative simplicity of these model communities with molecular and bioinformatics tools, the complex natural interactions could be deciphered one model community at a time. Ultimately, mixed cultures can be used to generate a working hypothesis to explore the microbial ecology and genetic population structures of the unseen vast majority of microorganisms.

KEY WORDS: Microbial ecology · Mixed cultures · Model communities · Cultivation · Interactions · Dependencies · Auxotrophy

INTRODUCTION

Microorganisms are the most diverse and abundant cellular life form on Earth (Rinke et al. 2013). Despite the great increase in the availability of diverse media and isolation techniques, a large majority of these organisms have not been cultivated (Amann et al. 1995, del Campo et al. 2016, Henson et al. 2016, Hug et al. 2016). In fact, we have only recently become aware of their presence, mainly through cultivation-independent molecular surveys based on ribosomal RNAs (Woese et al. 1990, Rajendhran & Gunasekaran 2011) or through shotgun sequencing (Gilbert & Dupont 2011). Ribosomal RNA analysis suggests that uncultivated organisms are found in nearly every prokaryotic group, and for several phyla no known cultivated representatives are known (Kaeberlein et al. 2002, Hug et al. 2016).

Until now, all these uncultivated microorganisms have been called 'unculturable.' However, 'unculturable' does not really mean that they cannot be cultivated; it rather indicates that current culturing techniques are unable to grow a given microorganism in pure cultures in the laboratory (Stewart 2012). Since most microorganisms do not easily grow in the laboratory, microbial ecologists have concentrated either on a subset of bacteria and archaea that are amenable to conventional culturing or on the use of culture-independent methods to describe microorganisms that compose natural microbial communi-

*Corresponding author: sarilog@gmail.com

Publisher: Inter-Research · www.int-res.com

ties. Nevertheless, cultivation remains important in the '-omics' age to validate genomic predictions and to allow the study of the organism as a whole (Giovannoni & Stingl 2007). A true understanding of the physiology of these bacteria and their roles in the environment (ecology), in host health, or in the production of metabolites requires that they be cultivated in the laboratory (Salcher & Šimek). In fact, cultivation-dependent and -independent methods should be seen as complementary and integrated approaches.

While heterotrophic flagellates have been cultured together with bacteria as their food (del Campo et al. 2013, Jeuck & Arndt 2013), bacteria have mainly been studied using Koch's classical cultivation methods, i.e. by growing isolated clonal populations from a single cell. Such isolation procedures aimed primarily at studying bacteria in relation to diseases and industrial processes (Penn & Dworkin 1976). Over the last century, microbiologists have isolated microorganisms from their natural communities and focused on their behavior in laboratory environments. However, even when the microorganisms have been taken away from their environment, the study of organisms in pure cultures has produced a staggering depth and breadth of knowledge in cellular microbiology. Nevertheless, the complexity of natural microbial communities demands that we direct our attention to more natural assemblages with genotypic and nutritional diversity (Little et al. 2008). Consequently, studying such assemblages will not only shed light on microbial interactions, but might also reveal the reason why obtaining pure cultures of many microorganisms is such a challenge.

In most cases, microorganisms exist in taxonomically diverse communities whose structure is determined by a complex interplay between environmental factors and ecological interactions among the respective community members (Little et al. 2008). Since many of those microorganisms still have not grown in pure culture in the laboratory, it is evident that the traditional isolation techniques used during the last 2 centuries are biased. First, because of the high nutrient density used in the media, we are biased towards cultivating copiotrophs. Second, the use of defined media under batch culture requires microorganisms, which were previously adapted to living in a complex community, to grow under certain new constraints: a limited variety of nutrient sources, a gradual decrease in the concentration of the nutrients, and a gradual increase in by-products. For instance, difficulties in isolating microorganisms relate to the use (quality and quantity) of organic matter, slow growth, oligotrophy, low cell densities, auxotrophy, unknown and/or unstable growth factors, dependence on the presence of other bacteria, and perhaps the combination of more than one of these reasons (Giovannoni & Stingl 2007, D'Onofrio et al. 2010, Carini et al. 2013, del Campo et al. 2013, Garcia et al. 2014).

Mixed cultures, as subsamples from complex natural communities, containing 2 or more bacterial strains can provide a simple enough community to rationally and robustly study and describe the individual community members (Fig. 1). At the same time, such mixed cultures share many properties with natural communities and hence could be used as model communities. Although cultures including multiple divergent phylotypes are often regarded as a failed attempt at classical pure-culture isolation (Brock 1987), some researchers have used mixed cultures as tools to study biochemical and ecological interactions or culture new microorganisms. In fact, mixed cultures are not a recent idea. In 1895, Sergei Winogradsky was the first to cultivate *Clostridium pasteurianum* through a series of mixed cultures, which were followed by pure culture experiments (Little et al. 2008). More recent examples include isolation approaches where mixed cultures have been more efficient for culturing bacteria indigenous to peat bogs (Dedysh et al. 2006), sediments (D'Onofrio et al. 2010), and marine (Morris et al. 2008) and freshwater systems (Hahn 2009, Jezbera et al. 2009, Garcia et al. 2014). Indeed, in many cases, cell material from mixed cultures can be used to attempt further isolation (Dedysh et al. 2006, Salcher & Šimek).

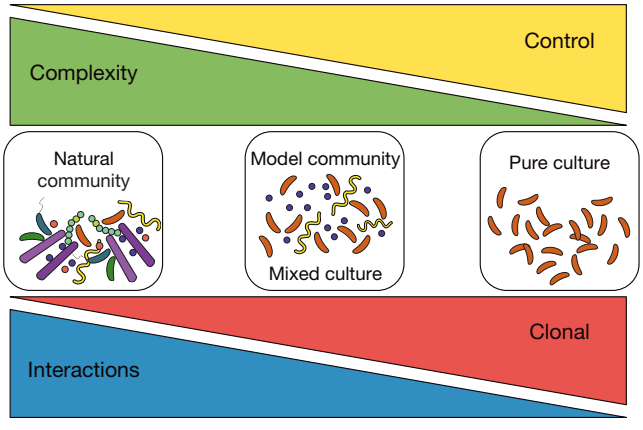

Fig. 1. Pros and cons of different approaches to the study of environmental microorganisms. Natural communities are complex with many interactions among their members. Pure cultures are clonal and allow for control of variables. Mixed cultures reduce the diversity of the natural community while still keeping some of the complexity, and can therefore be used as model communities

MIXED CULTURES AS TOOLS

Studying biochemical and ecological interactions

Mixed cultures have been widely used to study population interactions; for example, known microbial isolates have been mixed as a means of studying population dynamics such as competition and mutualism (Fredrickson 1977). Recently, synthetically mixed cultures have attracted interest as they can be screened for specific characteristics and functions which are useful for industrial applications such as fermentation, bioremediation, and production of biofuels (De Roy et al. 2014, Novoveska et al. 2016, Sargsyan et al. 2016). Moreover, studies of mixed cultures have already offered many examples of why growing microbial mixtures is a great starting point to study ecological interactions, search for specific biochemical transformations, and ultimately also cultivate those 'unculturable' microorganisms.

Several examples exist where some members of mixed cultures remove or utilize molecules that inhibit the growth of other community members (Fig. 2: cell A removes the inhibitor of cell B). Wilkinson et al. (1974), for instance, found that *Pseudomonas* sp. produces methanol from methane. In their study, this methanol was then consumed by *Hyphomicrobium* sp., which was also present in the community.

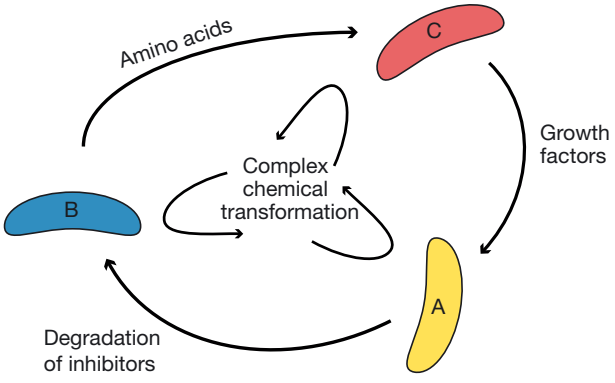

Fig. 2. Examples of interactions that could occur between different microorganisms (A, B, and C) in a mixed culture. Microbe A requires specific growth factors that can be provided by Microbe C. Also, as part of its metabolism, Microbe A uses/degrades a compound that inhibits growth of Microbe B. Hence, Microbe B can grow because Microbe A removes the inhibitor from its environment. Microbe B also produces all essential amino acids. Microbe C is auxotrophic for some essential amino acids that could be provided by Microbe B. Microbes A, B, and C together perform a complex chemical transformation. Some examples of these chemical transformations are mixed cultures performing anaerobic oxidation of methane (Boetius et al. 2000, Hu et al. 2009) and polyvinyl alcohol (PVA)-utilizing consortia (Sakazawa et al. 1981)

Methanol is strongly inhibitory to the growth of the *Pseudomonas*, but even at low concentrations, it supports rapid growth of *Hyphomicrobium*. It should be noted that the authors were not able to grow the focal *Pseudomonas* strain in pure culture (Wilkinson et al. 1974). Another more recent example of an inhibitory molecule being removed is found in mixed cultures of *Prochlorococcus*. After analyzing several mixed cultures and identifying the different possible helper bacteria, Morris et al. (2008) were able to purify the culture of *Prochlorococcus*. They speculated that helper bacteria were mainly removing hydrogen peroxide from the medium. Indeed, adding catalase enhanced the growth of the corresponding strain in pure culture (Morris et al. 2008). In a subsequent study, the same group demonstrated that removal of hydrogen peroxide is both necessary and sufficient for growth stimulation, thus corroborating the interpretation that the helper bacteria were reducing oxidative stress and thus allowing the sensitive *Prochlorococcus* to grow (Morris et al. 2011).

Mixed cultures have also been very useful when trying to disentangle chemical transformations that result from microbial metabolism (Fig. 2: cells A, B, and C working together). For example, anaerobic oxidation of methane is an environmentally important process mediated by microorganisms. Boetius et al. (2000) hypothesized that a consortium of archaea and sulfate-reducing bacteria performs the process in gas-hydrate-rich sediments. Ever since, many microbiologists have been trying to isolate the microbial strain that performs anaerobic methane oxidation, but instead, only mixed cultures have been obtained (Raghoebarsing et al. 2006, Hu et al. 2009). In another example, polyvinyl alcohol (PVA)-utilizing consortia were cultivated to show that PVA was used symbiotically by 2 bacterial members that could not use PVA when grown in monoculture (Sakazawa et al. 1981). One last example of chemical transformations is given by axenic and mixed cultures of *Rhodococcus rhodochrous.* These experiments testing the utilization of organosulfur compounds indicate that the desulfurization activity, on a per cell basis, is higher in mixed cultures than in axenic cultures (Kayser et al. 1993). Taken together, these examples highlight that mixed cultures can be used as models to study chemical transformations that are relevant in the environment.

Other interesting findings include the discovery of intercellular connections in mixed bacterial cultures. Pande et al. (2015) discovered that bacteria could connect to other bacterial cells via membrane-derived nanotubes — even in a well-mixed environment. Cells used these tubes to exchange cytoplas-

mic constituents, and their formation was induced by auxotrophy-causing mutations. Cells ceased to establish these connections when amino acids were externally supplied, thus uncoupling the otherwise obligate dependency among genotypes. These results showed that bacteria could use nanotubes to exchange nutrients among connected cells and thus help to distribute metabolic functions within microbial communities (Pande et al. 2015).

Mixed cultures are also highly relevant for drug research, because they allow identification of biologically active compounds (Netzker et al. 2015). This principle is nicely illustrated in a study in which the model fungus *Aspergillus nidulans* was co-cultivated with a collection of 58 soil-dwelling *Actinobacteria* (Schroeckh et al. 2009). It was found that a distinct fungal–bacterial interaction led to the specific activation of fungal secondary metabolism genes. These results provide strong evidence of specific interaction among different microorganisms and support the hypothesis that not only diffusible signals, but also intimate physical interactions, may contribute to the communication among different microorganisms and induce transcription of biosynthesis genes.

Uncultured bacteria are also quite common in host-associated environments, and mixed cultures can offer a chance to study host–microbe interactions (Stewart 2012, Krause et al. 2015). For example, co-cultures of algae and bacteria revealed that 2 specific bacterial strains (originally identified as *Roseobacter* sp. strain MS2 and *Cytophaga* sp. strain MS6) release diffusible, morphogenetic compounds that allow gametes of the marine green macroalga *Ulva mutabilis* to complete its normal development. In contrast, axenic cultivation of this macroalga resulted in slow-growing, callus-like colonies with aberrant cell walls (Grueneberg et al. 2016). Another example is the rice seedling blight fungus *Rhizopus microsporus* and its endosymbiont *Burkholderia rhizoxinica* that together form an unusual, highly specific alliance that produces the highly potent antimitotic phytotoxin rhizoxin (Moebius et al. 2014).

Summing up, mixed cultures have not only provided valuable insight into metabolic processes, ecological interactions, and nutrient cycling, but also into functional stability of ecosystems (Wittebolle et al. 2009). All of these recent examples illustrate how microorganisms grown in mixed cultures co-facilitate acquisition of necessary nutrients and growth factors, help metabolize inhibitors, and reduce stress, thereby improving the growth of all parties (Fig. 2). Hence it would be logical to use mixed cultures as a strategy to study microorganisms that elude cultivation in clonal fashion.

Studying population structures of microorganisms with streamlined genomes

Studying microorganisms in the context of a mixed culture becomes even more important considering that many free-living bacteria and archaea have streamlined genomes and are auxotrophic for one or more essential metabolites (Swan et al. 2013, D'Souza et al. 2014, Giovannoni et al. 2014). Streamlined genomes are typically characterized by (1) small genomes with highly conserved core genomes and few pseudogenes, (2) low ratios of intergenic spacer DNA to coding DNA, and (3) low numbers of paralogs. These streamlined genomes may provide prokaryotes with a competitive advantage in nutrient-poor environmental niches (Giovannoni et al. 2014). As a result of genome streamlining, gene loss renders free-living bacteria dependent on co-occurring microorganisms (or other environmental sources, e.g. plants, decaying organisms, etc.) to compensate the lost metabolic functions (Morris et al. 2012). Thus, mixed cultures could be powerful systems by resembling important features of the wild, while still reducing the complexity to manageable levels (Fig. 1).

The advent and development of culture-independent methods (Sanger et al. 1977, Woese et al. 1990, Hugenholtz et al. 1998, Tyson et al. 2004, Marcy et al. 2007, Woyke et al. 2009) opened up many exciting opportunities to study microbial communities. Moreover, applying bioinformatics tools to reconstruct genomes of previously uncultivated bacteria in communities with low microbial diversity has proven to be a successful approach (Tyson et al. 2004). In this context, mixed cultures have the low diversity necessary to make them tractable targets for genome reconstruction and associated studies of their metabolic potential.

For example, the abundant and ubiquitous freshwater *Actinobacteria* clade acI was first discovered in the environment during environmental ribosomal RNA surveys (Hiorns et al. 1997). This clade is very diverse, comprising 13 species or 'tribes.' Members of each tribe share ≥97% 16S rRNA gene sequence identity (Newton et al. 2011). Because acI constitutes up to 70% of total bacterial abundances in some freshwater systems (Warnecke et al. 2005), many microbiologists have tried to obtain acI in pure culture. Even though there has been reported successful isolation of a pure culture more than once and by more than one lab, for unknown reasons these cultures were unable to be propagated and died soon afterwards (H. P. Grossart, M. M. Salcher and F. Warnecke unpublished data). The first stable mixed

culture containing acI was named '*Candidatus* Planktophila limnetica' and only contributed <0.1% to the total cell numbers of culture when cultivated in NSY medium (Jezbera et al. 2009). Unfortunately, its low abundance did not enable much physiological insight into the lifestyle of these abundant players. Later, guided by cues provided by single-cell genomes (Garcia et al. 2013) (e.g. small genome size and lack of enzymes to reduce sulfur), efforts were made to culture acI in a mixed culture where acI was as abundant as in the environment. The mixed culture contained ~30% acI, grew successfully, and survived several transfers (Garcia et al. 2014). The culture in combination with qPCR thus made it possible to calculate doubling time (in the presence of the partners) of the acI. Moreover, the culture further illustrated the oligotrophic lifestyle of these streamlined bacteria. Furthermore, DNA was sequenced and the genomes of the main members of the mixed culture were characterized. Interestingly, 3 of the 4 members had no cultivated representative reported previously. Auxotrophy for several vitamins and amino acids was observed in the acI freshwater bacteria (and in all other members of the culture) as well as a genomic structure pointing to a possible division of labor (Garcia et al. 2015). This example further illustrates the power of mixed cultures to grow previously uncultivated bacteria. Mixed cultures have made it possible to study previously uncultivated bacteria, showing for most of them streamlined genomes as well as ecological interactions that allowed the members to metabolically complement each other.

CONCLUSION AND FUTURE PERSPECTIVES

Similar to conventional cultivation procedures for growing microorganisms in clonal cultures, work with mixed cultures is laborious and time-consuming (Ferguson et al. 1984, Eilers et al. 2000, Garcia et al. 2014). Moreover, the maintenance and stabilization of mixed cultures can be more challenging than the maintenance of pure cultures (Novoveska et al. 2016). However, a mixed culture is a first step to obtain a pure culture (Salcher & Šimek). In this way, the risk of ending empty-handed after hunting for a specific microorganism is lower than when aiming exclusively for a pure culture. Moreover, mixed cultures can be used to study ecological interactions among microorganisms in carefully controlled environments (Fig. 1). For these reasons, it would be of great benefit to adopt mixed cultures as part of the microbial ecologist's toolkit to cultivate microorganisms that have evaded clonal cultivation.

One drawback of using cultivated strains is that it is unknown whether they have evolved adaptations to the culture conditions or whether the initial cultivation has selected for a specific rare genotype (Giovannoni & Stingl 2007). However, if combining mixed culture strategy with DNA sequences from mixed cultures and environmental sources, the chances of understanding the genomic difference between the microorganisms obtained in the cultures and those inhabiting the environment are higher. Moreover, collecting several mixed cultures from the same environment could aid a more holistic understanding of the model communities that might uncover the complexity of the natural communities.

When working with mixed cultures, understanding the individual physiological features of the constituent community members is quite complex. However, mixed cultures can be used as sensors to fine-tune the development of artificial media that support the growth of the organisms of interest. Moreover, if several mixed cultures from the same environment could be analyzed, it might be possible to find the pattern of metabolites essentially required to grow and increase the chances of obtaining a pure culture. Accepting mixed cultures as a valid scientific method to study previously uncultivated bacteria will increase the number of microorganisms that we can study in culture, thus providing a chance to observe less biased samples of nature.

Acknowledgements. I thank Sari Peura, Moritz Buck, Stefan Bertilsson, and Christian Kost for helpful comments on earlier versions of the manuscript.

LITERATURE CITED

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Boetius A, Ravenschlag K, Schubert CJ, Rickert D and others (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407: 623–626

Brock TD (1987) The study of microorganisms in situ: progress and problems. In: Fletcher M, Gray TRG, Jones JG (eds) Ecology of microbial communities. Society for General Microbiology, Cambridge, p 1–17

Carini P, Steindler L, Beszteri S, Giovannoni SJ (2013) Nutrient requirements for growth of the extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium. ISME J 7:592–602

D'Onofrio A, Crawford JM, Stewart EJ, Witt K and others (2010) Siderophores from neighboring organisms promote the growth of uncultured bacteria. Chem Biol 17: 254–264

D'Souza G, Waschina S, Pande S, Bohl K, Kaleta C, Kost C (2014) Less is more: Selective advantages can explain the prevalent loss of biosynthetic genes in bacteria. Evolution 68:2559–2570

De Roy K, Marzorati M, Van den Abbeele P, Van de Wiele T, Boon N (2014) Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. Environ Microbiol 16:1472–1481

Dedysh SN, Pankratov TA, Belova SE, Kulichevskaya IS, Liesack W (2006) Phylogenetic analysis and *in situ* identification of bacteria community composition in an acidic sphagnum peat bog. Appl Environ Microbiol 72: 2110–2117

Del Campo J, Balague V, Forn I, Lekunberri I, Massana R (2013) Culturing bias in marine heterotrophic flagellates analyzed through seawater enrichment incubations. Microb Ecol 66:489–499

Del Campo J, Guillou L, Hehenberger E, Logares R, Lopez-Garcia P, Massana R (2016) Ecological and evolutionary significance of novel protist lineages. Eur J Protistol (in press)

Eilers H, Pernthaler J, Glockner FO, Amann R (2000) Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl Environ Microbiol 66:3044–3051

Ferguson RL, Buckley EN, Palumbo AV (1984) Response of marine bacterioplankton to differential filtration and confinement. Appl Environ Microbiol 47:49–55

Fredrickson AG (1977) Behavior of mixed cultures of microorganisms. Annu Rev Microbiol 31:63–87

Garcia SL, McMahon KD, Martinez-Garcia M, Srivastava A and others (2013) Metabolic potential of a single cell belonging to one of the most abundant lineages in freshwater bacterioplankton. ISME J 7:137–147

Garcia SL, McMahon KD, Grossart HP, Warnecke F (2014) Successful enrichment of the ubiquitous freshwater acI *Actinobacteria*. Environ Microbiol Rep 6:21–27

Garcia SL, Buck M, McMahon KD, Grossart HP, Eiler A, Warnecke F (2015) Auxotrophy and intrapopulation complementary in the 'interactome' of a cultivated freshwater model community. Mol Ecol 24:4449–4459

Gilbert JA, Dupont CL (2011) Microbial metagenomics: beyond the genome. Annu Rev Mar Sci 3:347–371

Giovannoni S, Stingl U (2007) The importance of culturing bacterioplankton in the 'omics' age. Nat Rev Microbiol 5: 820–826

Giovannoni SJ, Cameron Thrash J, Temperton B (2014) Implications of streamlining theory for microbial ecology. ISME J 8:1553–1565

Grueneberg J, Engelen AH, Costa R, Wichard T (2016) Macroalgal morphogenesis induced by waterborne compounds and bacteria in coastal seawater. PLOS ONE 11: e0146307

Hahn MW (2009) Description of seven candidate species affiliated with the phylum *Actinobacteria*, representing planktonic freshwater bacteria. Int J Syst Evol Microbiol 59:112–117

Henson MW, Pitre DM, Weckhorst JL, Lanclos VC, Webber AT, Thrash JC, Hallam SJ (2016) Artificial seawater media facilitate cultivating members of the microbial majority from the Gulf of Mexico. mSphere 1:e00028-16

Hiorns WD, Methé BA, Nierzwicki-Bauer SA, Zehr JP (1997) Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequences. Appl Environ Microbiol 63:2957–2960

Hu S, Zeng RJ, Burow LC, Lant P, Keller J, Yuan Z (2009) Enrichment of denitrifying anaerobic methane oxidizing microorganisms. Environ Microbiol Rep 1:377–384

Hug LA, Baker BJ, Anantharaman K, Brown CT and others (2016) A new view of the tree of life. Nat Microbiol 1: 16048

Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180:4765–4774

Jeuck A, Arndt H (2013) A short guide to common heterotrophic flagellates of freshwater habitats based on the morphology of living organisms. Protist 164:842–860

Jezbera J, Sharma AK, Brandt U, Doolittle WF, Hahn MW (2009) '*Candidatus* Planktophila limnetica', an actinobacterium representing one of the most numerically important taxa in freshwater bacterioplankton. Int J Syst Evol Microbiol 59:2864–2869

Kaeberlein T, Lewis K, Epstein SS (2002) Isolating 'uncultivable' microorganisms in pure culture in a simulated natural environment. Science 296:1127–1129

Kayser KJ, Bielaga-Jones BA, Jackowski K, Odusan O, Kilbane JJ (1993) Utilization of organosulphur compounds by axenic and mixed cultures of *Rhodococcus rhodochrous* IGTS8. J Gen Microbiol 139:3123–3129

Krause K, Henke C, Asiimwe T, Ulbricht A and others (2015) Biosynthesis and secretion of indole-3-acetic acid and its morphological effects on *Tricholoma vaccinum*-spruce ectomycorrhiza. Appl Environ Microbiol 81:7003–7011

Little AEF, Robinson CJ, Peterson SB, Raffa KE, Handelsman J (2008) Rules of engagement: interspecies interactions that regulate microbial communities. Annu Rev Microbiol 62:375–401

Marcy Y, Ouverney C, Bik EM, Lösekann T and others (2007) Dissecting biological 'dark matter' with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. Proc Natl Acad Sci USA 104: 11889–11894

Moebius N, Üzüm Z, Dijksterhuis J, Lackner G, Hertweck C (2014) Active invasion of bacteria into living fungal cells. eLife 3:e03007

Morris JJ, Kirkegaard R, Szul MJ, Johnson ZI, Zinser ER (2008) Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by 'helper' heterotrophic bacteria. Appl Environ Microbiol 74:4530–4534

Morris JJ, Johnson ZI, Szul MJ, Keller M, Zinser ER (2011) Dependence of the cyanobacterium *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface. PLOS ONE 6:e16805

Morris JJ, Lenski RE, Zinser ER (2012) The black queen hypothesis: evolution of dependencies through adaptive gene loss. MBio 3:e00036-12

Netzker T, Fischer J, Weber J, Mattern DJ and others (2015) Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. Front Microbiol 6:299

Newton RJ, Jones SE, Eiler A, McMahon KD, Bertilsson S (2011) A guide to the natural history of freshwater lake bacteria. Microbiol Mol Biol Rev 75:14–49

Novoveska L, Franks DT, Wulfers TA, Henley WJ (2016) Stabilizing continuous mixed cultures of microalgae. Algal Res 13:126–133

Pande S, Shitut S, Freund L, Westermann M and others (2015) Metabolic cross-feeding via intercellular nanotubes among bacteria. Nat Commun 6:6238

Penn M, Dworkin M (1976) Robert Koch and two visions of microbiology. Bacteriol Rev 40:276–283

Raghoebarsing AA, Pol A, van de Pas-Schoonen KT, Smolders AJ and others (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. Nature 440:918–921

Rajendhran J, Gunasekaran P (2011) Microbial phylogeny and diversity: small subunit ribosomal RNA sequence analysis and beyond. Microbiol Res 166:99–110

Rinke C, Schwientek P, Sczyrba A, Ivanova NN and others (2013) Insights into the phylogeny and coding potential of microbial dark matter. Nature 499:431–437

Sakazawa C, Shimao M, Taniguchi Y, Kato N (1981) Symbiotic utilization of polyvinyl alcohol by mixed cultures. Appl Environ Microbiol 41:261–267

Salcher MM, Šimek K (2016) Isolation and cultivation of freshwater microbes is essential for a comprehensive understanding of their ecology. Aquat Microb Ecol (in press), doi:103354/ame01796

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Sargsyan H, Trchounian K, Gabrielyan L, Trchounian A (2016) Novel approach of ethanol waste utilization: biohydrogen production by mixed cultures of dark- and photo-fermentative bacteria using distillers grains. Int J Hydrogen Energy 41:2377–2382

Schroeckh V, Scherlach K, Nutzmann HW, Shelest E and others (2009) Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. Proc Natl Acad Sci USA 106:14558–14563

Stewart EJ (2012) Growing unculturable bacteria. J Bacteriol 194:4151–4160

Swan BK, Tupper B, Sczyrba A, Lauro FM and others (2013) Prevalent genome streamlining and latitudinal divergence of planktonic bacteria in the surface ocean. Proc Natl Acad Sci USA 110:11463–11468

Tyson GW, Chapman J, Hugenholtz P, Allen EE and others (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43

Warnecke F, Sommaruga R, Sekar R, Hofer JS, Pernthaler J (2005) Abundances, identity, and growth state of Actinobacteria in mountain lakes of different UV transparency. Appl Environ Microbiol 71:5551–5559

Wilkinson TG, Topiwala HH, Hamer G (1974) Interactions in a mixed bacterial population growing on methane in continuous culture. Biotechnol Bioeng 16:41–59

Wittebolle L, Marzorati M, Clement L, Balloi A and others (2009) Initial community evenness favours functionality under selective stress. Nature 458:623–626

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87: 4576–4579

Woyke T, Xie G, Copeland A, González JM and others (2009) Assembling the marine metagenome, one cell at a time. PLOS ONE 4:e5299

Editorial responsibility: Paul del Giorgio, Montreal, Quebec, Canada

Submitted: February 24, 2016; Accepted: June 12, 2016
Proofs received from author(s): July 29, 2016