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Minireview

Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities

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Summary

Many microbial ecologists have described the composition of microbial communities in a plenitude of environments, which has greatly improved our basic understanding of microorganisms and ecosystems. However, the factors and processes that influence the behaviour and functionality of an ecosystem largely remain black boxes when using conventional approaches. Therefore, synthetic microbial ecology has gained a lot of interest in the last few years. Because of their reduced complexity and increased controllability, synthetic communities are often preferred over complex communities to examine ecological theories. They limit the factors that influence the microbial community to a minimum, allowing their management and identifying specific community responses. However, besides their use for basic research, synthetic ecosystems also found their way towards different applications, like industrial fermentation and bioremediation. Here, we review why and how synthetic microbial communities are applied for research purposes and for which applications they have been and could be successfully used.

Introduction

Microorganisms are ubiquitous on earth, with an estimated amount of 10^6 bacterial species (Lopez-Garcia and Moreira, 2008) and 4×10^{30} microbial cells globally (Horner-Devine *et al.*, 2004). Their genetic and physiological diversity result in an enormous metabolic potential. They contribute to nearly all biogeochemical cycles as they are the drivers of global and local nitrogen, oxygen, carbon, sulphur, and phosphorus cycles (Schmidt, 2006), what makes them essential for maintaining the Earth's biosphere and for the survival of plants and animals.

Most of these processes are accomplished by joint effort of microorganisms with different functional roles. These microorganisms do not act as individuals but rather act as a dynamically changing microbial community, where all cells interact and communicate with one another (Little *et al.*, 2008; Klitgord and Segre, 2010). They influence each other's behaviour and possibly alter the biochemical phenotypes of the participating strains (Wintermute and Silver, 2010).

Understanding the factors that shape and influence these microbial ecosystems is essential from a microbiological, ecological and biotechnological point of view. According to Prosser and colleagues (2007), this knowledge can be achieved by using a theory-driven approach: theories are generated based on existing observational data, after which they are verified using quantitative research. A deliberate choice of the experimental setup, methodology and microbial model systems is indispensable for optimal hypothesis testing. Pure cultures and complex microbial communities are conventionally used; however, synthetic ecosystems with intermediate complexity and high controllability are becoming increasingly popular.

From synthetic biology to synthetic microbial ecology

Culture-dependent methods allow the isolation of single microbial community members for in-depth analysis of their genetic and physiological characteristics. The body of literature on research with single microorganisms is tremendous (Jessup *et al.*, 2005). Since the -omics era, a lot of knowledge on these simple model systems is gained. Over 4000 complete microbial genomes have been sequenced, while more than 12 000 are in progress (http://www.genomesonline.org). Transcriptomics, proteomics and metabolomics gave further insight into their functionality, resistance to stress and adaptation. This increased understanding on how microorganisms

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function, led to the urge to steer and manipulate them. Synthetic biology, which is the application of engineering methodology to biology, was proven to be very useful (Endy, 2005; Leonard *et al*., 2008). Microorganisms have been engineered to improve their resistance to stress, to have a higher productivity and functional redundancy, to degrade toxic and recalcitrant compounds, to synthesize new chemical compounds, or to have other particular – unnatural – characteristics (Benner and Sismour, 2005). The numerous capacities of both genetically engineered and wild-type microorganisms make them interesting for different applications. They are used as probiotics in the medical and food industry (Steidler *et al*., 2000; Huibregtse *et al*., 2012), as cell factories for valuable products in the food, pharmaceutical, chemical and agriculture industry, with products ranging from anticancer drugs to biofuels (Du *et al*., 2011; Waegeman and Soetaert, 2011).

The fact that (i) only a small fraction of the microorganisms present in a microbial community can be cultured and (ii) the behaviour of microorganisms as pure cultures is different from their behaviour in a microbial community has caused a shift from single-organism studies to whole community studies. Molecular fingerprinting and high-throughput sequencing techniques are used to characterize these microbial communities. These techniques use a top-down approach and target microbial communities as a whole. Metagenomics, metatranscriptomics and metaproteomics give information on the taxonomic and functional diversity, the population structure, the presence of genes, as well as their levels of expression and translation into proteins (VerBerkmoes *et al*., 2009; Temperton and Giovannoni, 2012). A drawback is the complex post-processing of the big amount of data obtained by these high-throughput techniques (Raes and Bork, 2008). Even with the most advanced bioinformatics tools and sequencing technology, it is almost impossible to assign the (expressed) genes and proteins, and thus the functionality, to specific species (Temperton and Giovannoni, 2012; Zengler and Palsson, 2012). Furthermore, it is not possible to fully map and understand the microbial interactions, which are often the driving force of a community.

Compared with the amount of literature available on single organisms and complex microbial communities, only a small fraction of microbial ecology research makes use of synthetic microbial communities. Synthetic microbial ecology is a collective term for all rationally designed ecosystems that are created by a bottom-up approach where two or more defined microbial populations are assembled in a well-characterized and controlled environment (Fig. 1). These synthetic ecosystems have a lower complexity, higher controllability, higher reproducibility and are a simplified representation or simulation of natural ecosystems. Synthetic ecosystems are used (i) to gain insight in fundamental principles such as metabolic processes, interactions, networking, diversity-functionality relation and nutrient cycling, and (ii) to create interactions and communities with desired characteristics and functionality. Alternative terms for similar experimental setups are microcosms or artificial ecosystem, while other terms have been mistakenly used for synthetic ecology: (i) synthetic biology, which is the engineering of cells, and (ii) systems biology, which considers the use of a top-down approach to understand a system by characterizing the different parts.

Synthetic microbial ecology for theory testing

While a microbial community as such is already complex, numerous environmental factors further increase the level of complexity (Fig. 2). Microorganisms live in close contact with each other as they continuously **interact and communicate (A)** with one another (Little *et al*., 2008; Klitgord and Segre, 2010). These interactions may be unidirectional or bidirectional (West *et al*., 2006). Molecules are produced that can be beneficial or detrimental for both the actor and recipient. Different kinds of interactions and cooperation are present in nature: mutualism, syntrophy or cross-feeding (beneficial to the actor/beneficial to the recipient; +/+), selfishness (beneficial to the actor/costly to the recipient; +/–), spite (–/–), and altruism (–/+) or parasitism (+/–) (West *et al*., 2007; Faust and Raes, 2012). Microorganisms can communicate with one another through mechanisms like quorum-sensing, which allow them to express certain genes only under favourable circumstances (Manefield and Turner, 2002). Next to the abundant microorganisms that actively contribute to the functionality of the ecosystems, numerous species are present in lower abundance. They are regularly categorized as redundant and are responsible for the **resilience (E)** of the community (Bissett *et al*., 2013). **Abiotic factors (C)** like temperature, salinity and pH can alter the environment in such a way that they cannot perform their role in the community anymore (Wu and Conrad, 2001; Sharma *et al*., 2006). Under these circumstances, redundant species can take over and guarantee the ecosystem functionality. The resilience of a community is thus also strongly dependent on the **community diversity (B)** (Loreau *et al*., 2001). Both the number of microorganisms (richness) and their relative abundance (evenness) influence the resistance to stress, invasion and predation (Wittebolle *et al*., 2009; Saleem *et al*., 2012; De Roy *et al*., 2013). Next to the microbial diversity, also the **spatial organization (F)** as it exists in a biofilm, can be of importance (Tolker-Nielsen and Molin, 2000). It allows only those species that are located in close proximity to interact and communicate with each other; furthermore, it provides microenvironments and niches for specific microbes.

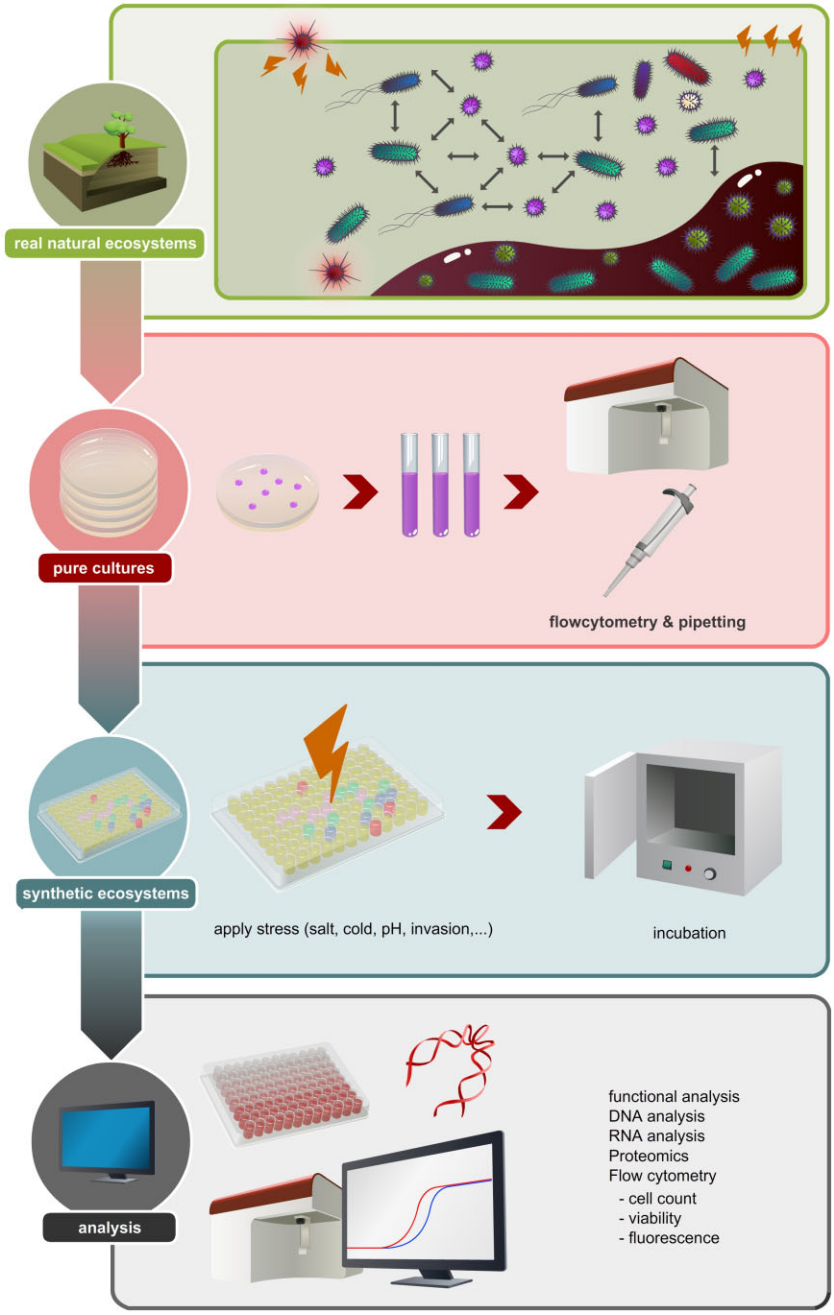

Fig. 1. Strategy of how to create synthetic ecosystems. Synthetic communities are created by a bottom-up approach. This includes that microorganisms are initially isolated from their natural environment via conventional culture-based techniques. Upon growth in liquid media, they are quantified via flow cytometry and diluted to the desired cell numbers. Synthetic communities are then created by mixing microbial species in specific proportions under desired conditions, after which they are incubated. Finally, all parameters of interest, like functionality and cell count, are analysed.

All these factors shape, characterize and influence an ecosystem and its functioning. By interfering with one of these parameters, a complete ecosystem might collapse. However, also the opposite might happen as an ecosystem may perform better or new functions can be introduced. By doing research and gaining knowledge on these fundamental principles, it will become possible to steer, manage and create ecosystems to optimize their performance.

In situ or *in vivo* models are complex systems in which nearly all of the earlier mentioned influencing factors are present, thus giving a good representation of the real situation. The complexity of the microbiota in these systems is useful for the validation of different products or treatments but may also be a confounding factor for research purposes, as most of the influencing factors are hard to control. Intrinsic system effects and reciprocal interactions may even lead to opposite conclusions on the role of a specific parameter in closely related ecosystems (Wilsey and Polley, 2002; Emery and Gross, 2007). For this reason, synthetic ecosystems are a powerful tool to investigate fundamental principles in natural and

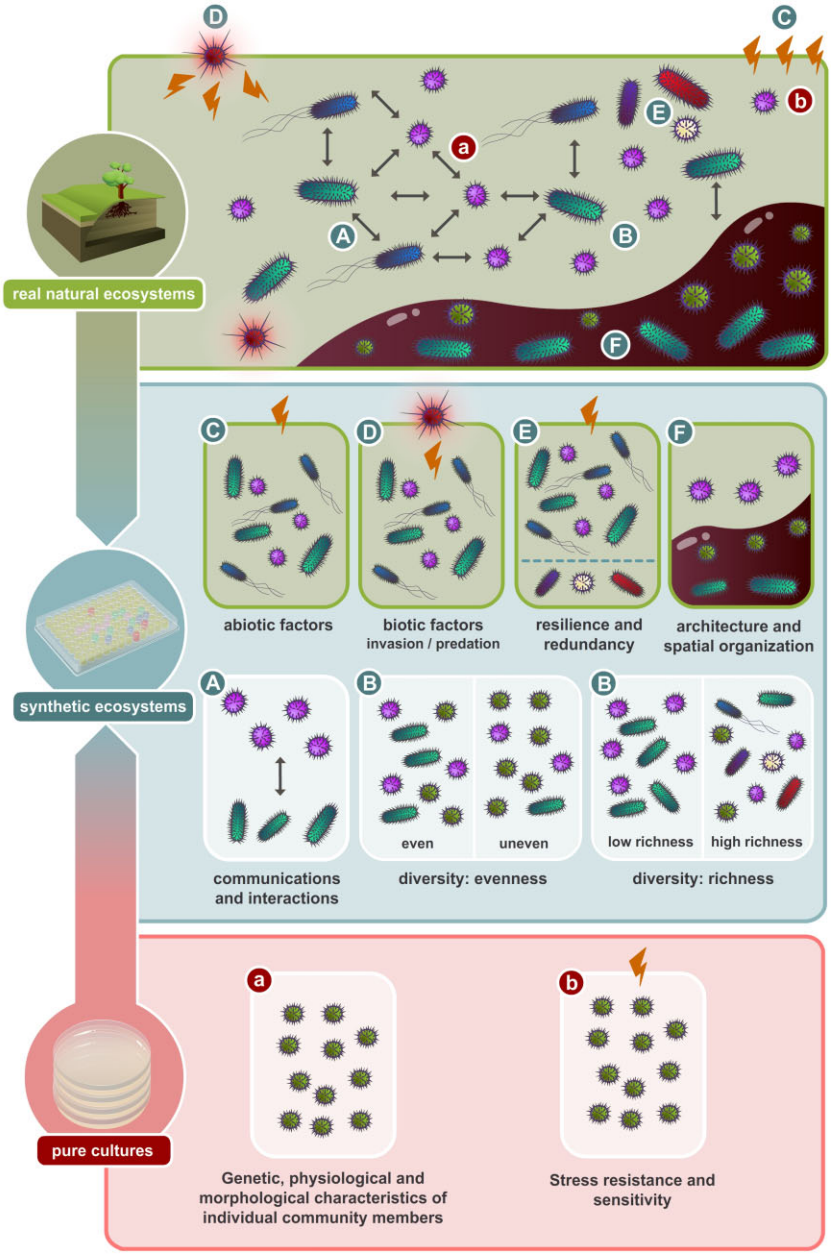

Fig. 2. Synthetic ecosystems for research purposes. Natural ecosystems are complex as many factors influence and shape microbial communities. These factors are included: (A) microbial metabolic interactions, signalling and communication; (B) diversity; (C) abiotic or environmental factors; (D) biotic factors like invasion and predation; (E) resilience and redundancy; and (F) architecture and spatial organization. Research with pure cultures provides information on genetic, physiological and morphological characteristics of specific microbes (a), as well as on their resistance and sensitivity to stress (b). However, they do not allow researchers to investigate the factors that shape and influence microbial communities. For this purpose, synthetic ecosystems are a powerful tool as they have a reduced complexity and higher controllability compared with natural ecosystems. They also allow to focus on specific parameters of interest while excluding other influencing factors.

engineered systems. They limit the influencing factors to a minimum, allowing their management and tracking of the effects of the earlier mentioned parameters. Furthermore, fully characterized microorganisms with a well-defined genetic background can be used in synthetic ecosystems. In the following paragraphs, we provide several examples of how synthetic microbial ecosystems have been used to study the role of specific influencing factors.

The first synthetic ecosystems were used to study microbial interactions and signalling, as reviewed by Yu and colleagues (2012). For this type of research, communities mainly consist of only two or three microbial species, which are often also being genetically engineered to create the interaction of interest or to simplify tracking of the parameters of interest. In this way, hypotheses can be tested that would otherwise not be accessible (Wintermute and Silver, 2011). Next to creating an interacting community by genetically engineering the organisms, Klitgord and Segre (2010) showed that it is also possible to create interactions by changing the environment: for every two species-consortia, a cooperation-inducing environment could be identified. Environmental factors, like the availability of nutrients, temperature, presence of toxic compounds and oxygen-level not only influence microbial interactions but also influence the resilience of a community, which on its turn

is influenced by the microbial diversity. To get insight in the biodiversity–productivity relationship along different kinds of stress, researchers also opted for synthetic microbial ecosystem experiments. This allows controlling the evenness and richness, the applied stress and the follow-up of the functionality, which is not possible in natural environments. Doing so, Wittebolle and colleagues (2009) investigated the effect of community evenness on the functionality of a denitrifying bacterial community in the presence and absence of salinity stress. They created over 1000 synthetic ecosystems in 96-well plates with the same 18 denitrifying strains but with different levels of initial evenness. It was concluded that highly uneven communities (low biodiversity) are less resistant to environmental stress than even communities (high biodiversity). The latter could better retain their functionality under stress conditions. In another study regarding the effect of richness on resistance to cadmium pollution, 330 synthetic ecosystems characterized by changing numbers of algal species were created. It was shown that the conservation of biodiversity (richness) may reduce the future impacts of increasing environmental stresses (Li *et al*., 2010). A positive relationship between richness and functionality was also shown by Bell and colleagues (2005) by using synthetic microcosms with up to 72 bacterial species. Finally, Gravel and colleagues (2011) showed that the loss of specialists – strains that exploit only few resources – has a stronger effect on ecosystem functioning compared with loss of generalists, which are able to use a spectrum of substrates.

The effect of trophic interactions – such as predation – on ecosystem functioning was investigated by altering the predator and prey richness. Predators were simulated by three bacterivorous protists, while five bacterial strains were used as model organisms of the prey. It was shown that the presence of multiple predators resulted in increased bacterial diversity, which had a positive effect on bacterial yields (Saleem *et al*., 2012; 2013).

As the effect of invasion is mainly studied during observational studies in natural ecosystems, many controversies on the outcome of invasion exist (Lambertini *et al*., 2011; Lockwood *et al*., 2011). By using more than 3000 synthetic ecosystems, it was show by De Roy and colleagues (2013) that the contradicting results can be explained by the environmental condition under which invasion occurs. In the absence of salt stress, invasion by non-native species in an uneven community had adverse effects on the community functionality. In contrast under stress, invasion of the same strain can help the community to perform better. Invasion was also shown to be higher in uneven communities compared with even communities in the absence of salt stress. On the contrary, evenness has no effect on invasion in the presence of stress. The importance of the environment on interactions between different species was also shown by Hu and colleagues (2010). By using two quorum-sensing circuits, they designed a synthetic ecosystem in which different antibiotic and initial cell density levels resulted in different interactions and population dynamics, such as extinction and mutualism.

Finally, the spatial organization and architecture of microbial communities is also crucial to maintain a stable and functional community. By combining FISH (fluorescent *in situ* hybridization) with a digital image analysis software that quantifies the spatial localization patterns of microorganisms in complex samples, it was shown that functionally linked species cluster together in a microbial community (Daims *et al*., 2006). Kim and colleagues (2008) controlled the spatial organization of a community by using a microfluidic device that controls the distance between three wild-type soil bacterial populations with syntrophic interactions. In this community, each species is required for the survival of the community. It was shown that spatial organization is necessary to balance competition and beneficial interactions to create a stable community (Kim *et al*., 2008). Brenner and Arnold (2011) used two genetically engineered *Escherichia coli* populations to study the benefits of the formation of physical structures like biofilms. Species associated in a biofilm were shown to be more productive than non-associated community members.

In conclusion, the use of synthetic ecosystems increased our knowledge regarding factors that shape and influence microbial communities. Such advances would have been difficult to obtain in natural ecosystems because of the presence of confounding factors that are hard to control or measure. As a result, the research regarding synthetic ecosystems initiates many opportunities to manage ecosystems. By changing one of the parameters, the community can be steered, and a desired effect can be created. This approach is generally known as microbial resource management (MRM) and will be elaborated in the following section.

Synthetic communities for applications

MRM has been defined as the optimal management of microbial resources in order to develop novel products and processes to improve the environment or human health in the most sustainable way (Verstraete *et al*., 2007; Read *et al*., 2011). Management may occur at the level of single cells, i.e. engineering of individual microbial populations to improve their resistance to stress, to have a higher productivity or to degrade toxic compounds (Benner and Sismour, 2005). Furthermore, management may also occur at the level of the complex microbial community, which inhabits natural and anthropogenic environments and whose final functionalities often result

from metabolic networking among the different members. As described earlier, both extremes have distinct advantages and disadvantages. Next to research purposes, synthetic ecology can therefore be of importance for the development of specific applications, representing a good balance in terms of complexity, relevance and manageability.

Synthetic communities can be used, for instance, to recycle waste products. The European Space Agency designed MELiSSA (Micro-Ecological Life Support System Alternative), a bioregenerative life support system for the complete recycling of gas, liquid and solid wastes during long-distance space exploration (Fulget *et al*., 1999; Hendrickx *et al*., 2006). In MELiSSA, cyanobacteria and plants are use as food sources. As both cyanobacteria and plants preferentially take up nitrogen as nitrate, the ammonium-enriched liquid waste derived from human activities needs to be nitrified to nitrate to create the most optimal recycling system. Therefore, ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (i.e. genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*) and then nitrite to nitrate by nitrite oxidizers (i.e. genera *Nitrobacter*, *Nitrococcus* and *Nitrospira*). Considering that MELiSSA has been designed for space exploration, the stability of the system is a key aspect in order to assure long-term functionality. In this respect, the choice of a synthetic community should assure both a functional and compositional stability as the environment is well-defined and the required metabolic conversions are not complex. In fact, according to Pimm (1984), the more the functionality of one species depends on the activity of another species, the fewer species will be necessary to maintain ecosystem stability. Moreover, as the loss of a species would lead to the disruption of the whole ecosystem, the designed synthetic community should be also resilient to perturbation (Pimm, 1984).

Synthetic communities also play a key role in the industrial fermentation and production of chemical compounds. In industrial bioethanol production, most ethanol is produced by the fermentation of glucose or sucrose from corn, sugar cane or beets. Because this competes with food production, alternative sources of sugar are investigated, such as lignocellulosic biomass. Glucose and xylose are the two dominant sugars. But current approaches are inefficient because no native microorganisms can convert all sugars into ethanol at high yield. Therefore co-cultures of strains that have a high yield for different sugars are used (Chen, 2011). Patle and Lal (2007) showed that a very simple community composed of *Zymomonas mobilis* and *Candida tropicalis* was able to transform enzymatically hydrolysed lignocellulosic biomass into ethanol with a yield of 97.7%. Mixed-culture fermentation from lignocellulosic biomass for ethanol production can increase ethanol yield and production rate, and reduce process cost.

Synthetic microbial communities consisting of *Ketogulonicigenium vulgare* and *Bacillus megaterium* have been used in industry to produce 2-keto-gulonic acid (2-KGA), the precursor of vitamin C (Ma *et al*., 2011). By means of quantitative systems biology analysis, it was shown that the cell lysis of *B. megaterium* provided key elements necessary for *K. vulgare* to grow better and produce more 2-KGA as compared with the production as a pure strain. Also, Masset *et al*. (2012) demonstrated the benefits of working with a synthetic community as compared with pure strains in the field of hydrogen production from starch. Traditionally, pure strains give better H_2-yields as compared with mixed communities. However, the main limitation of this approach is the need to work under sterile conditions. Communities composed by *Clostridium pasteurianum* and *C. felsineum* and by *C. butyricum* and *C. pasteurianum* were shown to offer better performance in terms of H_2 production from different carbon sources than the single strains. Moreover, in contrast with the pure cultures, the co-cultures were able to use starch without any need for prehydrolysis.

Another field of application for synthetic communities is the bioremediation of contaminated areas. This approach often relies on the addition of microorganisms with the metabolic potential to degrade a specific contaminant, i.e. bioaugmentation. Given the high complexity of some contaminants, bioaugmentation of single strains may not be sufficient to achieve a good 'removal efficiency', as demonstrated in the case of the pesticide linuron (Dejonghe *et al*., 2003). *Variovorax* sp. strain WDL1 could degrade linuron using it as C, N and energy source. Conversely, *Delftia acidovorans* WDL34 and *Pseudomonas* sp. strain WDL5 were not able to use linuron but only some intermediate of its degradation. When these strains were mixed in a synthetic community, the rate of linuron degradation improved due to the synergistic interaction of the strain WDL1 with the other bacteria. A similar case is represented by the degradation of 4-chlorosalicylate. This compound can only be degraded if *Pseudomonas reinekei* (MT1), *Wautersiella falsenii* (MT2), *Achromobacter spanius* (MT3) and *P. veronii* (MT4) work together (Pawelczyk *et al*., 2008).

A final example is the application of synthetic microbial communities as a safe alternative for human faecal transplants. Because the human gut contains a dense (10^{13}–10^{14} microbial cells) and diverse microbial community (Eckburg *et al*., 2005), consisting of several hundred microbial species, severe disturbances of this ecosystem are unlikely to be resolved by the administration of a single probiotic strain. Indeed, recurrent *C. difficile*-associated diarrhoea (Khoruts *et al*., 2010; Guo *et al*., 2012), which is thought to result from persistent disruption

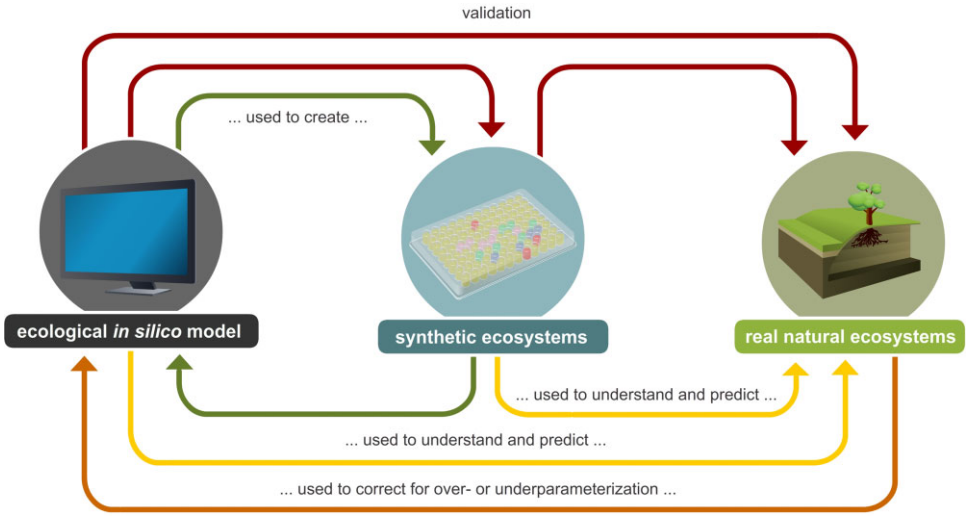

Fig. 3. The future of synthetic ecosystem research. Research with synthetic ecosystems drastically increased the knowledge on microbial ecosystems. All this information could be used to create *in silico* models that can predict an ecosystem's behaviour. After validation and correction for possible overparameterization or underparameterization, these models could be used to understand, predict, manage and create ecosystems.

of the commensal gut microbiota, was cured upon transplantation of a complex faecal microbiota derived from a healthy human donor (Shahinas *et al.*, 2012). This approach is however only applied in severe cases given the high complexity of a human faecal sample, which is inherently associated with a certain risk for transmitting disease. As a result, there is a large potential for synthetic ecology to mix a well-characterized and safe set of gut microorganisms. Petrof and colleagues (2013) synthesized a synthetic microbiota consisting of 33 individual microbial species and indeed demonstrated the potential of such synthetic microbiota in the eradication of *C. difficile* infections. Such approaches may result in a replacement of commonly used antibiotics.

All the cases described in this section demonstrate the potential that synthetic communities may cover in practical applications. Despite this potential, the road to translate MRM into practice is still long, and several aspects require further investigation, as outlined later.

Future perspectives

The majority of synthetic ecosystems consist of only two to four species. Although being very useful to study ecological theories, the resemblance with natural ecosystems and potential for practical applications may be more limited. Therefore, a next step in synthetic ecology is to create synthetic ecosystems with increasing resemblance to natural ecosystem. The better a model can simulate the actual complexity of nature, the higher its scientific value. First, this can be achieved by using sophisticated experimental models that better simulate the environmental factors. An example of a sophisticated model is a high-pressure reactor to simulate the deep-sea environment (Zhang *et al.*, 2011). Second, synthetic ecosystems can be optimized by increasing the number of species and optimizing their composition, structure and functionality. Such studies have mostly been restricted to short-term experiments because of stability issues of synthetic communities. It was theoretically shown by ecological models that some species and specific mixtures of agonistic and mutualistic interactions between species are necessary to obtain a stable ecosystem (Boyd, 2012; Mougi and Kondoh, 2012). The integration of such models in microbial ecology would be of high value. Research with synthetic microbial ecosystems created an enormous amount of complementary data; in addition, the genomes of numerous microorganisms have been sequenced. By combining these data and information, *in silico* models making use of 'digital microorganisms' could be created and used for the construction of synthetic ecosystems with desired characteristics (Fig. 3) (Yedid *et al.*, 2009). Furthermore, these models could be used to predict an ecosystem's behaviour like stability, resistance and functionality. The problem with many ecological models is the lack of validation and overparameterization. Therefore, we argue to use real ecosystem, *in vivo* models, sophisticated *in vitro* models or synthetic ecosystems for the validation of *in silico* theoretical models and correct for possible overparameterization. But also to use real ecosystems to check the relevance of synthetic ecosystems because numerous important factors could be missed.

Only when this is done, models can really contribute to the understanding, prediction and management of ecosystems.

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