MINI-REVIEW

Microbial Resource Management revisited: successful parameters and new concepts

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Abstract In the twenty-first century, scientists will want to steer the microbial black box in (engineered) ecosystems, rather than only study and describe them. This strategy led to a new way of thinking: Microbial Resource Management (MRM). For the last few years, MRM has been utilized to consolidate and communicate our acquired knowledge of the microbiome to many areas of the scientific community. This shared knowledge has brought us closer to formulating a plan toward the analysis, and at a later stage, the management of our varied microbial communities and to look at ways of harnessing their unique abilities for future practices. We require this acquired knowledge for a more sustainable solution to our ongoing global challenges such as our diminishing energy and water supply. Like any successful concept, MRM must be updated to adapt to new molecular technologies, and thus, in this review, MRM has been reengineered to encompass these changes. This review reports how MRM has been used successfully over the last few years within various environments and how we can broaden its capabilities to increase its compliance in the face of state of the art ever changing technologies. Not only have we reengineered and improved MRM, but also we have discussed how newly formed relationships between technologies can provide the full picture of these complex microbial communities and their interactions for future opportunities.

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Introduction

Microbial communities are ubiquitous in nature, and yet, the complex interrelationships within them and how environmental fluctuations affect these communities still elude us. This knowledge is essential as the "super challenges" of the twenty-first century (Verstraete et al. 2007) increase, our resources continue to decrease, the climate change debate heightens, and diseases still threaten our existence. Microorganisms can exist in association without distinctive interactions with each other or the host which we call *microbiota*. Alternatively, when the microorganisms cooperate with each other and show specific interactions, such as cross signaling, cross feeding, aggregation, and adhesion, the term "microbiome" is used. Microbiome indicates a group of microorganisms which no longer exist at random and have a higher level of organization and functionality. Consequently, the need to understand the "microbiome," while simultaneously being able to manage them, may provide us with a more sustainable solution for our ongoing challenges.

Four years ago, with this in mind, Verstraete et al. (2007) developed the Microbial Resource Management (MRM) concept. This is a practical mindset, as shown by the MRM flow sheet in Fig. 1, and has been developed as a concept to solve practical problems through the use of microorganisms. This flow sheet demonstrates how to logically determine the correct direction to proceed when implementing the big picture of MRM. In the future, it could

Fig. 1 MRM flow sheet demonstrating how to logically determine the correct direction to proceed when implementing the whole MRM concept
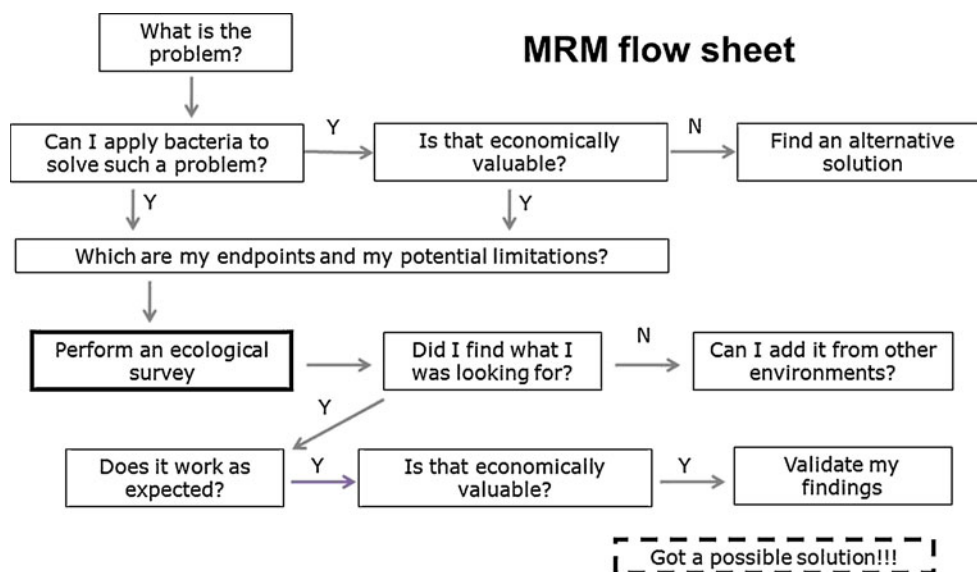
provide research, engineers, and policy makers with a new mindset in the context of microbial ecology and environmental microbiology. The key proposed purpose is to control and/or steer the microbial capabilities associated to complex microbial communities. In order to manage, it is fundamental to find an answer to straightforward questions, like "who is there," "who is doing what," and "who is with whom." As a consequence, the ecological survey in Fig. 1 is a key step in the decisional flow. For this reason, the inception of MRM was shortly followed by a complementary set of tools to deal with it (Marzorati et al. 2008) which come in the form of a three-stage analysis independent of the technique and its settings. These tools help to provide an ecological and predictive value of the analysis which incorporates the structure and diversity of the microbial community being examined. The growing area of microbial ecology has presented us with many new developments, thus requiring an overview and re-evaluation of the MRM concept and its tool set.

In this review, we will only discuss the tools based on microbial analysis that support the MRM concept. Initially, a three-stage tool set was based on 16S rRNA gene molecular fingerprinting pattern interpretation, specifically denaturing gradient gel electrophoresis (DGGE; Marzorati et al. 2008). The first part of this review assesses how MRM has been used over the last few years for different molecular techniques and proposes what it can offer for the future. Additionally, we have also investigated how the MRM tool set can be adapted to encompass new techniques such as high-throughput sequencing and the relationships being formed between technologies such as stable isotope probing (SIP) and three-dimensional (3D) imaging. By identifying the strengths and weaknesses of the MRM tool set, we have, where necessary, reengineered its components to establish a more robust platform suited to our constantly changing environment and evolving technology. The reader should address the text with an eye to the past

(what has been done) and one to the future (new theoretical concepts that will require validation in practice).

MRM revisited

The most popular molecular fingerprinting techniques including temperature gradient gel electrophoresis (TGGE) and DGGE, terminal-restriction fragment length polymorphism (t-RFLP), 16S rRNAgene clone libraries, and length heterogeneity-polymerase chain reaction (LH-PCR) are commonly used to study the structure and composition of the microbial communities. Interpreting and comparing this type of data became easier due to the initiation of MRM and the three-stage tool set developed by Marzorati et al. (2008). The parameters within the tool set included range-weighted richness (Rr), dynamics (Dy), and functional organization (Fo). When using these tools in combination, they can provide us with an ecological interpretation of the raw data describing the structure of the community. This has been demonstrated successfully in various environments over the last few years including the human gut (Grootaert et al. 2009; van den Abbeele et al. 2010); wastewater treatment (Wittebolle et al. 2009b, c; Wittebolle et al. 2008; Vlaeminck et al. 2009); prebiotics and human gut microbial diversity(Marzorati et al. 2010b; Possemiers et al. 2010); microbial community related to celiac health issues (Schippa et al. 2010); drinking water (Lautenschlager et al. 2010); anaerobic digestion (Carballa et al. 2010; Pycke et al. 2010); aquaculture (Qi et al. 2009; De Schryver et al. 2010; De Schryver et al. 2011; Crab et al. 2009; Prol-Garcia et al. 2010); poly-β-hydroxybutyrate (PHB)-accumulating reactors (Morgan-Sagastume et al. 2010); and microbial fuel cells (MFC; Aelterman et al. 2008; Freguia et al. 2010) just to mention a few (Table 1).

Table 1 Previous studies which have used the MRM tool set in their analysis, including the environment investigated, method used, goals of their study, and specific MRM results

Environment	Method	MRM focus/goal	Rr	Dy	Fo	Reference
Wastewater	DGGE	Evaluating reactor functionality to nitrification efficiency related -AOB community	Medium–high	Medium	Medium	Wittebolle et al. 2009c
	DGGE	Improve and optimize wastewater treatment processes through more rigorous methods to monitor a control -AOB community	High	High	Medium	Wittebolle et al. 2009b
	DGGE	Nitrogen removal from black water-AOB community	Low	Not analyzed	Medium	Vlaeminck et al. 2009
	RISA	Changes in available nutrients in extreme soils	High	Not analyzed	Not analyzed	Rojas-Oropeza et al. 2010
	TGGE	Optimization of wastewater treatment systems and solving process failures	Not suited to Archaea[a]	Not analyzed	Medium for bacteria, high for Archaea	Gomez-Silvan et al. 2010
Microbial fuel cells	DGGE	Harvesting energy from the wasted sludge of domestic wastewater	High	Not analyzed	Low	Freguia et al. 2010
	DGGE	Increasing electricity generation through the use of different electrode materials	High	Not analyzed	Not analyzed	Aelterman et al. 2008
Drinking water	DGGE	Monitoring of drinking water in household taps	Low	Not analyzed	Low	Lautenschlager et al. 2010
Biodegradation	PCR/RISA	Bioaugmentation of activated sludge to remove 3-chloroaniline	Not analyzed	Medium	Not analyzed	Bathe et al. 2009
	DGGE	Removal of 1,2-dichloroethane using anodophilic microbial consortia	Established acetate fed MFC-closed circuit-high, open circuit-med-high/newly started MFC-closed and open circuit-low	High	High to medium	Pham et al. 2009
Anaerobic digestion	Clone library	Bioremediation of an aquifer contaminated with 1,2-dichloroethane	High	Not analyzed	High	Marzorati et al. 2010a
	T-RFLP	Performance of four full-scale anaerobic digesters and their unique microbial communities	High	High	High	Pycke et al. 2010
	DGGE/T-RFLP	Wastewater treatment and recovery of valuable resources using anaerobic digestion	High	Bacteria higher Dy than Archaea	Archaea higher Fo than bacteria	Carballa et al. 2010
Human gut	DGGE	Effect of different prebiotics on the human intestinal microbial ecosystem	High	High	Medium	Grootaert et al. 2009
	DGGE	Modulation of the human gut microbial community by plant-derived polysaccharide-rich dietary supplements	Bifidobacteria-high	High	Low	Marzorati et al. 2010b
	DGGE	Evaluation of chocolate as a carrier for probiotic	High	High	Not analyzed	Possemiers et al. 2010
	DGGE/HITChip	Evaluation of a tool to study the mechanism of action of bacteria in the human gut	Medium	Not analyzed	Medium	Van den Abbeele et al. 2010
	TTGE	New therapeutic strategies to treat patients with celiac disease-restoring the intestinal ecosystem	High	Not analyzed	Not analyzed	Schippa et al. 2010
Aquaculture	DGGE	Overcoming wintering problems for tilapia hybrid fingerlings	Not analyzed	Not analyzed	Medium	Crab et al. 2009
	DGGE	Increased growth of rotifer production in marine fish hatcheries	Not analyzed	Not analyzed	Varied evenness-strain dependent	Qi et al. 2009
	DGGE	Use of Poly-β-hydroxybutyrate to increase the growth performance of juvenile European sea bass	High	Not analyzed	Not analyzed	De Schryver et al. 2010
	DGGE	Increasing intestinal health of juvenile European sea bass using PHB	Medium–high	Low	Low	De Schryver et al. 2011
	DGGE	Develop effective prevention strategies of fish larvae infection	Low	Not analyzed	High	Prol-Garcia et al. 2010
Poly-β-hydroxybutyrate (PHB) accumulating reactors	DGGE	Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream	Not analyzed	Not analyzed	High	Morgan-Sagastume et al. 2010

Rr—Low (<10%), Medium (10–30%), High (>30%)

Dy—Low (<7%), Medium (7–24%), High (>24%)

Fo—Low Fo and high evenness (25%), Medium Fo and evenness (30–70%), High Fo and low evenness (70%)

[a] The range of values proposed for bacteria needs to be modified when working with Archaea

These abovementioned studies, which commonly used fingerprinting techniques such as DGGE, fatty acid methyl ester (FAME), clone libraries, and t-RFLP, have helped us to elucidate unknown characteristics of natural prokaryotic ecosystems in these various areas utilizing MRM. The first parameter, Rr, was originally introduced to establish a technique-specific range of values which indicate the richness and genetic diversity (based on the polymorphism of sequence of the 16S rRNA gene) of species within an indigenous bacterial community (Marzorati et al. 2008). Rr was based on the DGGE gel patterns derived from the GC content and positioning of the sequences from complex microbial communities. Hence, the information contained in positioning, percentage, and stretches of GC within the 16S rRNA gene were used to mathematically express Rr. The latter was expressed as the total number of bands multiplied by the percentage of denaturing gradient needed to describe the diversity of the sample being analyzed. The formula was:

$$Rr = \left(N^2 \times Dg\right) \qquad (1)$$

where N represented the total number of bands in the pattern, while Dg was the denaturing gradient comprised between the first and last band of the pattern. A high Rr value indicates an environment with a high carrying capacity, an environment that can host several species with a wide GC variability.

This parameter, although technique-dependent, can still be used to assess community-based fingerprinting techniques. For example, it has been successfully used with rRNA intergenic spacer analysis (RISA; Rojas-Oropeza et al. 2010), TGGE (Schippa et al. 2010), and clone libraries (Marzorati et al. 2010a). In contrast, Gomez-Silvan et al. (2010) highlighted in their study that Rr is not a suitable parameter when analyzing the Archaeal community due to the difficulty in extracting a substantial amount of sequence data. This is problematic when dealing with Archaeal areas of research as there is a lack of specific and reliable primers because of unavailable sequence data and hence, a circular problem. Notwithstanding, Rr has been successfully used in determining the richness of communities in various environments as shown in Table 1.

Additionally, these results are comparable between related studies, specifically those involving the human gut, MFC, and wastewater areas. For example, Freguia et al. (Freguia et al. 2010) and Aelterman et al. (Aelterman et al. 2008) both observed a high Rr within their MFC experiments. When determining Rr, Freguia et al. observed that the acetate-fed communities showed a higher performance and were also clustered together (giving low Gini values) indicating a high evenness and thus high community Rr. Similarly, Aelterman et al. also observed a high Rr

value (above 30) which translates into a competent environment with a good carrying capacity.

Dy, the second parameter in the tool set, was used to determine the rate of change within the same community over a fixed time interval. It refers to the number of species, on average, that are detected to be of significance in a given environment at a certain time point, thus providing a big picture of the dynamics within a community. The Dy parameter was initially calculated through processing DGGE patterns to calculate a matrix of similarities for the densiometric curves of the DGGE patterns based on the Pearson product-moment correlation coefficients. The similarity percentage values can be recalculated to percentage of change values using the following formula:

$$\% \text{ change} = 100\% - \text{similarity} \qquad (2)$$

The moving window analysis is used to determine the % change value matrix by plotting values between consecutive sampling points. These determine the rate of change (Δt) value that can provide the average of the respective moving window curve of the data points. Thus, the higher Δt is then correlated to the higher moving window curve data point and therefore the higher changes between the DGGE profiles within two consecutive time points. In addition, Dy can be used as a standalone parameter as seen previously in a study looking at the changing community during bioaugmentation of activated sludge (Bathe et al. 2009).

Table 1 shows the resilience of Dy as it allows comparisons between studies of a similar nature. Dy has also been seen to paint a very nice story of microbial community dynamics as seen in the study of Pham et al. (2009) where closed and open circuit MFC were used to remove 1,2-dichloroethane by anodophilic microbial consortia. This study showed that an acetate-fed community changed more quickly than that of the original inoculum during the removal of 1,2-dichloroethane. This also resulted in higher reactor performance indicating a more stable community able to adapt to changes within its environment. Similarly, a study by Marzorati et al. (2010b), observing changes in the human gut microbial community during treatments of plant derived polysaccharide-rich dietary supplements, observed the change in community dynamics using the Dy parameter in conjunction with the suit of the MRM tool set. These two examples demonstrate the successful implementation of Dy within two different settings providing unequivocal results for the conclusions of their studies.

The third complementary parameter is the functional organization (Fo; Marzorati et al. 2008). This parameter initially was designed to determine the resulting action of which microorganisms were suited to the ongoing environ-

mental–microbiological interactions. This should inevitably give them a selective advantage against the other bacteria, thus increasing their dominance among the other species in the microbial community being examined. Species distribution was visualized using the Lorenz evenness curves (Lorenz 1905) which was derived from DGGE profiles. The Lorenz curve graphically depicts the distribution relationship between species richness and abundance and can be used to describe the relative abundance of the community (Naeem 2009). DGGE bands were ranked from high to low based on their intensities, and the cumulative normalized bands were used as the x-axis, while their normalized intensities formed the y-axis. The more deviation of the Lorenz curve from the 45° diagonal indicates less evenness in the structure of the community due to the fact that a small number of species are dominant and many others resilient.

In a recent paper of Wittebolle et al. (2009a), it was elegantly demonstrated how initial evenness within the structure of a microbial community gives more resistance to change in function during times of environmental stress. Previous studies have shown how richness affects the community function and structure (Cardinale et al. 2002; Loreau and Hector 2001); however, this study was the first to observe how the initial community evenness is integral in maintaining functional stability and resilience. These results have given us a further understanding of biodiversity and how an ecosystem's functioning and stability can be affected through environmental fluctuations while also demonstrating the importance of the Fo parameter.

Similarly, Fo was successfully used to demonstrate changes in evenness in various areas of research including wastewater and MFCs. In particular, a study by Freguia et al. (2010) demonstrated that through the use of the complete MRM tool set, different communities evolved depending on the volatile fatty acid (VFA) that was present. In this study, a high evenness was found during those experiments using acetate, indicating that the community is well structured to deal with change which was also linked to successful reactor performance.

Another interesting observation was seen in two previous studies by Wittebolle et al. (2009c, b) where medium values of Fo (according to the range reported in Marzorati et al. (2008)) were linked to a high Rr which has also been reiterated in previous studies, suggesting functional redundancy (Tilman 1996; Bell et al. 2005). These medium Fo results demonstrated that although only a small amount of AOBs were dominant, this did not affect the system during change indicating functional stability within the community. This suggested that a pool of AOB species are capable of replacing the dominant AOB species and performing the necessary function when required, thus leading us to the assumption of functional redundancy.

Fo was initially thought of as a way to correlate structure and functionality of the microbial community. However, fingerprinting techniques largely make use of the differences on the 16S rRNA gene to discriminate among different bacterial species. In this respect, it is not always possible to correlate a given functionality with the respective group of microorganisms at the 16S level. As a consequence, Fo fails the original purpose of correlating the distribution of bacteria and their respective role in the overall functionality.

As we have already seen (Wittebolle et al. 2009a), initial community evenness is integral in maintaining functional stability and resilience during environmental fluctuations, but it is not always possible to make this correlation. In consideration of this issue, we have decided to rename Fo as community organization (Co), as a parameter that describes the microbial community in terms of degrees of evenness. Fo was originally correlated to the Gini coefficient, a value describing the degree of evenness within a community by measuring the normalized area between the Lorenz curve and the perfect evenness line. A high level of unevenness in a community is indicated by the higher Gini value (Wittebolle et al. 2009a). Co also describes the species abundance distribution in the microbial community, has a percent value between 0 and 100 (0=even community, 100=uneven community), and is calculated as the Gini coefficient times 100.

The Fo concept can still be used for functional analysis of a sample but only when the fingerprinting technique is based on functional genes that allow the analysis of the phylogeny within a microbial community (e.g., denitrification genes or *dsr* gene for sulfate-reducing bacteria). This is the ideal case as it is possible to correlate the structure of a microbial community to the stability of a specific functionality.

Adaptation of the MRM toolbox to the new molecular toolbox

The recent development of new technologies providing high-throughput, low-cost sequencing methods has provided us with alternatives including Lynx Therapeutics' Massively Parallel Signature Sequencing (MPSS; Reinartz et al. 2002), 454 pyrosequencing (Edwards et al. 2006), Illumina (Solexa) sequencing (Whiteford et al. 2009), and ABI SOLiD (Sequencing by Oligonucleotide Ligation and Detection) sequencing (Valouev et al. 2008). These technologies are more sensitive than the traditional DGGE and other fingerprinting methods and provide us with a broader taxonomic coverage of the unknown and often unculturable microbial communities. Therefore, the basic parameters of the MRM tool set have to be reengineered and adapted to

provide a universal platform with which to compare and contrast this new immense amount of data. Rr, based on community fingerprinting methods, was often limited in comparing richness in complex communities due to their low detection limit (Bent et al. 2007). These techniques often considerably underestimate the actual richness of communities and hence the actual diversity, which is why we must now be more vigilant in the face of new sequencing technologies. In this respect, the Rr value for sequencing-based techniques should include both the estimated number of unique operational taxonomic units (OTUs) by using rarefaction curve analyses and the phylogenetic broadness of the present OTU's on a family, class, or order level. On the contrary, Dy is an ageless parameter, which will easily adapt to the introduction of any new analysis methods and technologies and hence does not need any specific modification.

Finally, the coparameter needed to be adapted to be more responsive when analyzing large datasets. In fact, high-throughput sequencing provides a much deeper insight of the actual diversity of the analyzed microbial community. As a result, the analysis provides not only the sequences of the dominant microorganisms but also of all those less abundant microorganisms normally not detected with the abovementioned fingerprinting techniques due to the low detection limit. This normally "unseen majority" in turn will consequently alter the shape of the Lorenz curve and the relative Gini coefficient toward complete dominance, thus rendering the Lorenz curve calculation of little use. To encompass the addition of the rare species, we propose a new strategy. Consider a dataset of N OTU classes. Initially, all OTU classes are sorted from high to low abundance (with OTU-1 having the most sequences and OTU-N the fewest). Secondly, the different Co (C_o^i, with i=1 to N) values are calculated for a progressing window of OTUs, starting from 1 to N. These C_o^i values can then be plotted against the amount of classes (i). This new curve will then describe how quickly a microbial community is reaching its maximum Co value (C_o^∞). This was tested using four 454-datasets from different environments (unpublished data). These included samples from an enhanced biological phosphorous removal reactor (EBPR), an anaerobic biore-actor, a biological activated carbon filter, and a sand filter. Figure 2 demonstrates that these datasets show different trend lines with the graphs for all samples reaching a different final Co representing the degree of evenness. For example, the EBPR reactor quickly reaches a Co value of 90, meaning that this microbial community is strongly dominated by a few species. On the other hand, the microbial community from the anaerobic bioreactor stabil-izes at a Co value of 55, representing a balanced microbial community. Moreover, the shape of the line, besides the final Co value, contains information about the microbial
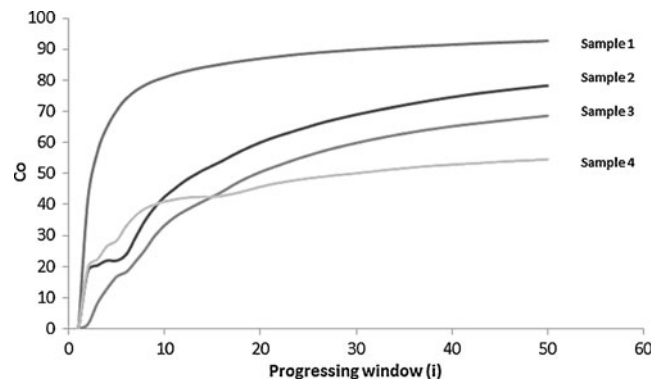

Fig. 2 This graph, of unpublished 454 data, shows evenness of values calculated for a progressing window of OTUs, starting from 1 to N. i varied from 1 to 50 in this example, and these C_o^i values can then be plotted against the amount of classes (i) which will then describe how quickly a microbial community is reaching its maximum Co value (C_o^∞). Samples are 1 enhanced biological phosphorous removal reactor (EBPR), 2 anaerobic bioreactor, 3 biological activated carbon filter, and 4 sample from a sand filter

community composition and more specifically, about the relative abundance of the most dominant species. The higher the unevenness, the more quickly the final Co value will be reached. For example, comparing the anaerobic bioreactor and the biological activated carbon filter, not only is the final Co value approximately the same, but also the left side of the trend lines clearly differ. Further ecological interpretation is needed to validate this new approach using the progressing window of OTUs.

New concepts and parameters for MRM

MRM has generated a lot of interest over the years, and scientists have applied the parameters in various ways to suit their environment (Table 1). What has become clear is that we still need to focus on identifying the typical characteristics and benchmark values of every environ-ment. This information would then enable the use of these parameters in practical applications. In other words, once we know what is "normal," we can use MRM to predict the reaction of a given environment. By combining this knowledge with new technologies, we could then eluci-date previously concealed interrelationship within the microbiome.

Since Edwards et al. (2006) completed the first meta-genome analysis using pyrosequencing and compared the results to a clone library study, it was obvious that a lot of important information about microbial communities was being excluded. Now, with all this additional information, interpretation and analysis must also be extended and capable of accommodating this abundance of extra infor-mation. Although the MRM tools are still valuable, as

shown above, they must be able to adequately deal with the added value of high-throughput sequencing and be able to link to other types of analysis providing the full picture of the community and its changes. With this in mind, we would like to introduce new concepts within the MRM tool set that could help in providing a further ecological interpretation: Ecological Pareto, Community distortion factor, and Architecture parameter.

Ecological Pareto and Community distortion factor

Benchmark values are indispensable when assessing and comparing the efficiencies of engineered water treatment systems. In this framework, we already proposed to use the Community organization index. Besides, we also proposed that the economical law of Pareto (distribution of wealth in the population) could also apply in microbial ecology (Verstraete et al. 2007). Here, to describe the optimal community organization of an ecosystem, we are introducing a new concept, the Ecological Pareto (Ep). Ep is a value that considers the optimal microbial community organization for a certain environment, which can differ from ecosystem to ecosystem and needs to be determined as demonstrated in our theoretical environment in Fig. 3. We could argue that less or nonfunctional ecosystems will deviate from this Ep at a certain degree (Fig. 4), and therefore, the severity of this distortion can be described by the Community distortion factor (Cd). The Cd parameter reflects the degree to which the community structure is different from the most optimal community structure. In essence, the absolute values of Cd describe the severity of the distortion while the +/− indicates, respectively, a too uneven (+) or a too even (−) community (Fig. 5).

Fig. 4 This graph uses a theoretical sample to demonstrate the distortion of a Community organization (Co) in relation to the Ecological Pareto (Ep) using Lorenz curves showing. In this case, the community is too even

This descriptive Cd value can be calculated as follows: If Co<Ep, the community is too even

$$Cd = \left(\frac{Co - Ep}{Ep}\right) \times 100 => \text{value between} -100 \text{ and } 0 \quad (3)$$

If Co>Ep, the community is too uneven

$$Cd = \left(\frac{Co - Ep}{100 - Ep}\right) \times 100 => \text{value between } 0 \text{ and } 100 \quad (4)$$
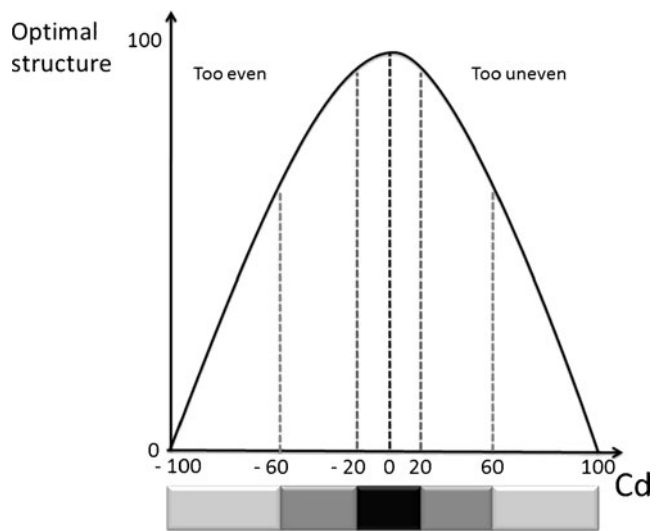
Fig. 5 A theoretical environment is used in this graph to demonstrate the Community distortion (Cd) of a certain Community organization (Co) in relation to the Ecological Pareto (Ep). The absolute values of Cd are describing the severity of the distortion, the +/− indicates respectively a too uneven or a too even community. The degree of distortion is divided into three classes: no distortion (between 0% and 20%), partial distortion (between 20% and 60%), and severe distortion (between 60% and 100%)
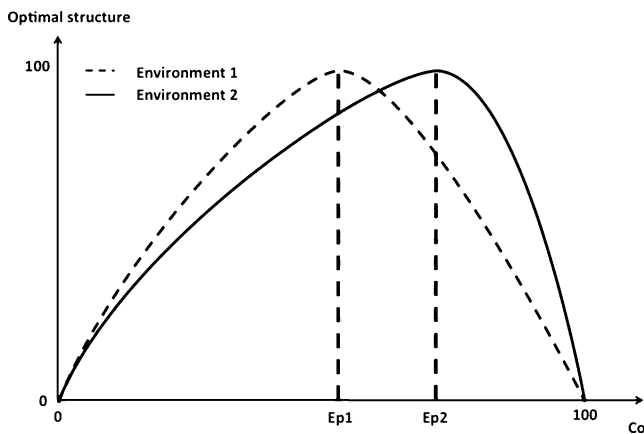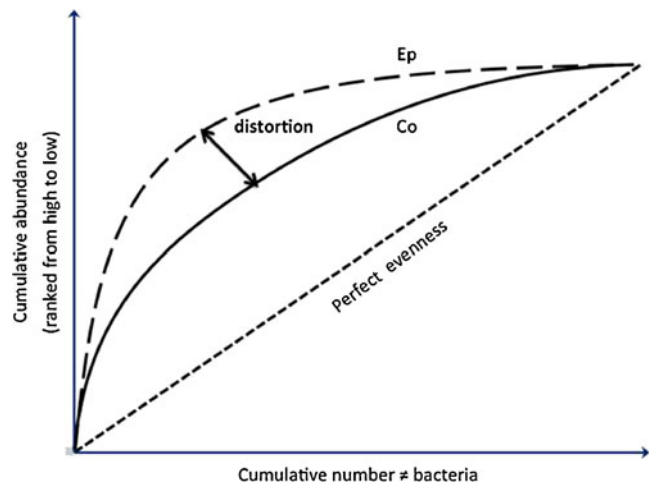

Fig. 3 This graph contains two theoretical environments which demonstrate the optimal structure of an ecosystem determined by its Community organization (Co). This value is the Ecological Pareto (Ep) in this theoretical example

The resulting graph of the Cd value is a normalized value, where we can have some set points for orientation as visualized in Fig. 5. We divided the degree of distortion into three classes: no distortion (between 0% and 20%), partial distortion (between 20% and 60%), and severe distortion (between 60% and 100%).

Evaluation of the 3D structure and an Architecture parameter

Results from sequencing and fingerprinting techniques analyzed through software such as BioNumerics software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium) can be further related to the 3D structure of the community using software such as Daime (Daims et al. 2006) or COMSTAT (Heydorn et al. 2000). This 3D information can be gathered through the use of fluorescent in situ hybridization (FISH; Wagner et al. 1994) in conjunction with confocal laser scanning microscopy (CLSM). This not only provides us with changes in the community from a genomic/species perspective and with a partial answer to the question "who is with whom" but also allows us to see the physical changes within the community by observing how a community alters its architectural structure to maintain functionality in the face of environmental stress or alterations.

Structural architecture refers to the position of the component elements in a 3D arrangement. It is already known that the structure of sludge flocs, granules, and biofilms is very porous, and the bacteria inside are considered to undergo the same nutrient flow compared to suspended bacteria (Logan and Hunt 1988). Subsequently, these structures cannot be seen as dense solid particles of microorganisms (Smith and Coakley 1984; Li and Ganczarczyk 1988) and gradients of substrates that exist (Wu et al. 1987), and the cells are entrapped in extracellular polymeric substances (EPS) layers. FISH techniques or labeling bacteria with fluorescent proteins (Eberl et al. 1997; Wagner et al. 1994) in conjunction with CLSM can reveal the structural organization; thus, in order to take into account all these issues, we are proposing to introduce the Architecture parameter (Ar).

The computer program, COMSTAT, is an excellent tool to quantify 3D biofilm image Z stacks (Heydorn et al. 2000). Four different parameters can be calculated providing meaningful interpretation to the architecture of the community. Firstly, biomass density or biodensity (cell dry weight/μm^3) represents the overall density of the biofilm while also providing an estimate of the biomass in the biofilm. Secondly, porosity is the number and area size of microcolonies at the substratum providing valuable infor-

mation about the organization of the biofilm community. Additionally, the distribution of microcolony volumes may be interesting in many respects, e.g., to investigate how microcolony volumes vary under changing environmental conditions, or to establish whether there exists an "optimal microcolony volume," so that microcolony growth ceases when a certain microcolony size is reached.

Thirdly, thickness distribution can be used to calculate a range of variables, including mean biofilm thickness and roughness coefficient (Heydorn et al. 2000). Mean biofilm thickness provides a measure of the spatial size of the biofilm and is the most common variable used in biofilm literature, while the dimensionless roughness coefficient is calculated from the thickness distribution of the biofilm. Biofilm roughness provides a measure of how much the thickness of the biofilm varies, and is also an indicator of biofilm heterogeneity. Lastly, distribution of diffusion distances is also an important parameter when analyzing biofilms. Average and maximum diffusion distances have been suggested as measures of the distances over which nutrients and other substrate components have to diffuse from the voids to the bacteria within microcolonies. The distribution of 3D diffusion distances in biofilms can be used for detailed studies on diffusion in biofilms. Average and maximum diffusion distances can be used as simple variables characterizing the "diffusion state" of the biofilm. One last area that should also be included in the study of biofilms is the level of extracellular architectural material which can be calculated based on specific fluorescent staining techniques for EPS (Bahulikar and Kroth 2007) and extracellular DNA (Steinberger and Holden 2005; Bockelmann et al. 2006).

Another alternative or complementary biofilm tool is the Daime software (Daims et al. 2006). Daime has the ability to find two-dimensional (2D) and 3D objects in images, quantify microbial populations, and quantify spatial localization patterns of microorganisms in complex samples using FISH images or other staining techniques. The use of quantitative FISH analysis and 3D imaging in conjunction with DGGE analysis using the MRM tool set has been successfully used over the last couple of years (Wittebolle et al. 2009c; Vlaeminck et al. 2009). The inclusion of 3D biofilms analysis was comprehensively demonstrated in a recent study by Vlaeminck et al. (2009) where they observed changes in an ammonia-oxidizing bacteria (AOB) community through the transition from a synthetic medium to digested black water. During this study, they were able to observe the spatial arrangement of the biofilm community using FISH and 3D analysis which was nicely linked to the DGGE and MRM analysis. These results verified the dominant species to be aerobic AOBs and produced a comprehensive picture of the entire study.

MRM and SIP

A final technique to understand "who is doing what" and to observe the specific community dynamics is the SIP combined with DGGE. SIP is based on an ultracentrifugation step that fractionates a microbial sample previously incubated with the compound of interest which was isotopically labeled (Neufeld et al. 2007). The result is a separation of the microbial sample according to its weight, which is dependent on its isotopic content. SIP is an excellent technique to identify key microbial players in mixed communities and to look at a specific process under a controlled environment. With the key players identified, their physiology and ecology can be investigated, therefore providing important tools for MRM.

Through SIP, we can determine which microorganisms are responsible for the process of interest in addition to interactions within the community providing us with information on the functional organization of the community. As mentioned above, we are replacing the Fo parameter with Co due to experimental limitations; however, when using SIP, the functionality of the microbial community is extensively analyzed and can be correlated with the community diversity. Therefore, in such cases, the Co parameter should be referred as CoF to clarify its specificity. Besides calculating the CoF value, we can also use the Dy parameter in combination with SIP to estimate the networking potential of a microbial community. Indeed, when a ^{13}C-labeled compound is fed to a microbial community, it will be consumed by a limited amount of species. However, the more metabolites are exchanged with other microbial members, the more additional bacteria will be labeled. As such, when the Dy parameter is calculated on ^{13}C-labeled rRNA with a time window of an hour, we expect that in a community exchanging substrates, the Dy will be much higher than in a community where the substrate is only consumed by a limited amount of species not producing and excreting any metabolites.

Conclusions and future perspective

Microorganisms are an almost unlimited source of metabolic capabilities ready to be exploited for multiple purposes (production, health benefit, bioremediation just to mention a few). As shown in Fig. 1, this is a quite laborious process but, once successfully implemented, can transform an ecological problem into an economically valuable proposition. In order to exploit these avenues, it is first important to understand how this complex world works and which rules bacteria respond to. For this reason, our revised MRM tool set (Table 2) is fundamental even if it is just one part of the entire MRM mindset.

Table 2 Definitions of the all MRM parameters

MRM parameters	
Range-weighted richness (Rr)	Introduced to establish a technique specific range of values which indicate the richness and genetic diversity (based on the polymorphism of sequence of the 16S rRNA gene) of species within an indigenous bacterial community
Dynamics (Dy)	Used to determine the rate of change within the same community over a fixed time interval
Community organization (Co)	Describes the species abundance distribution in the microbial community and is calculated as the Gini coefficient times 100
Community organization and function (CoF)	Used when functionality is still being examined eg. when using SIP
Ecological Pareto (Ep)	A value that considers the optimal microbial community organization for a certain environment
Community distortion factor (Cd)	Reflects the degree to which the community structure is different from the most optimal community structure
Architecture parameter (Ar)	Structural parameter is very important for activated sludge flocs, granules and biofilms, it is rarely quantified
C_o^∞	How quickly a microbial community is reaching its maximum Co value
C_o^i	The different Co (C_o^i with $i=1$ to N) values are calculated for a progressing window of OTUs, starting from 1 to N. These values can then be plotted against the amount of classes (i)

As shown in Table 1, the MRM tool set has been successfully used throughout the last years in different experimental settings with varying techniques. Results from these studies show a trend within similar environments which again reiterates the robustness of the MRM concept and its tool set. On the other hand, although in some cases these concepts have been brought to practice, most of the time their use has been limited to the simple characterization of a given environment. Nowadays, we are at a very important crossroad for microbial ecology wherein through the development of relationships between technologies and theories we will be able to elucidate the complexities of biodiversity within communities. This will allow the establishment of links between microbial community identification (working as whole entities) and their metabolic function. The incorporation of the new MRM tools will bring about an interesting novelty in order to define default values for different environments, conditions, and values that will act as yardsticks for the actual management of these complex ecosystems. Here, we proposed new

concepts that, when successfully used throughout the next years, can inspire researchers within this field. The road to success is under construction.

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