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The Role of Microbial Community Engineering (MCE) in the Water-Energy-Food-Waste (WEFW) Nexus

Carlos E. Gómez-Camacho*, Francesca Bosco, Bernardo Ruggeri*

Politecnico di Torino, Dep. of Applied Science and Technology (DISAT), C/so Duca degli Abruzzi 24, 10129 Torino.

carlos.gomezcamacho@polito.it and bernardo.ruggeri@polito.it

Microbial community engineering (MCE) is a growing branch of the biotechnological field, which uses co-cultures or mixed cultures of different strains or species to exploit the natural diversity and interactions among microbial groups. Urgent concerns regarding the Water-Energy-Food-Waste (WEFW) nexus started to raise a couple of years ago, while biological systems have long addressed the nexus issues for their survival since ancient times. The availability of carbon (food/waste) and energy sources (solar or chemical), as well as water activity in specific environments, has shaped through evolution the metabolism of individual and mixed consortia of microorganisms. The accumulation of metabolic products or the depletion of substrates in a given natural niche can potentially modify the environmental conditions and hence promote population selection shifts. Microbial consortia present species–species and species–environment interactions, which can exert selective pressure and influence the complex interplay of the biological system. Microbial communities sustain a range of ecosystem services, including freshwater supply, carbon and nitrogen cycles, human health, bioenergy production, CO₂ sequestration and bioremediation. The present review addresses higher-order properties of microbial communities, such as interspecies electron transfer and quorum sensing, highlighting some of the available applications and tools of MCE in the context of the WEFW nexus.

1. Introduction

The awareness for the water-energy-food-waste (WEFW) nexus has recently received increasing interest from different stakeholders in multi-disciplinary projects. The approach of the Food and Agriculture Organization (FAO) of the United Nations considers a “*holistic vision of sustainability that recognises and tries to strike balance between the different goals, interests and needs of people and the environment*” (FAO, 2014).

The underlying question is, however, whether economic models, production and consumption patterns, can be coupled to natural cycles; in simple terms, to keep track of societal metabolism. In this respect, the FAO approach focuses primarily on the water-energy-food (WEF), which can be considered valid but misses a key pillar of the nexus: waste. Climate change, for example, is considered in FAO’s approach to be a driver, while it must be considered a consequence of neglecting the role of waste within the strategies to manage nexus, hence a wider perspective should instead address the water-energy-food-waste (WEFW) nexus.

One of the most stringent consequences of the mismanagement of the WEFW nexus and the lack of integrated strategies is the alarming loss of biodiversity and the endanger of different forms of life. The agricultural sector, for example, has focused its production using mainly five types of livestock in the food industry: pigs, cattle, poultry, sheep and goats. In 2017, the European Union reported 150.5, 88.4, 2 223.6, 86.8 and 12.7 million heads (Eurostat, 2018), respectively, while the human population amounted to c. 500 million inhabitants; this poses a strong pressure on the environment in terms of biodiversity, organic wastes and energy consumption. However, not only higher trophic life forms should be considered for a comprehensive analysis, but also the vast microbial biodiversity that plays a fundamental role in biogeochemical cycles. Indeed, these cycles are the result of the interaction of the biosphere with different reservoirs, such as the atmosphere, hydrosphere and lithosphere; in particular, the cycles of key elements such as C, N, S, P, O, H and Fe and their role in the

structuring and degradation of complex biopolymers, such as polynucleotides, polypeptides and polysaccharides.

Microorganisms live in all environments on earth that are occupied by higher trophic organisms but do also thrive in harsh and extreme environments, where macroscopic life is not found. Although a thorough quantification of microbial species in natural environments might be impossible to perform, (Flemming and Wuertz, 2019) recently hypothesized that most bacteria and archaea on Earth (1.2×10^{30} cells) can be found in communities and aggregated in the form of biofilms, in four dominant habitats: deep oceanic subsurface (4×10^{29}), upper oceanic sediment (5×10^{28}), deep continental subsurface (3×10^{29}) and soils (3×10^{29}) while in oceans (1×10^{29}) single-cell is believed to be the dominant form. In natural environments, microbial communities address the WEFW nexus in a different way compared to individual strains and taxa of microbes acting alone or than that cultivated under controlled bioreactors conditions. The differences seem to arise from emergent higher-order features that provide insights on how to improve and exploit the potential of biotechnological applications using MCE. In agricultural systems, soil, plants and livestock microbial diversity can be used to shed light on ecosystem functioning, which can significantly improve the overall agroecosystem activity. In environmental applications, bacterial and fungal microbial communities have proved their potential in the bioremediation of different type of contaminations and bioenergy production. This paper examines some key features which can be of interest to understand the dynamics of complex microbial ecosystems aimed to help the development of the next-generation bioprocesses and to exploit the potentiality of microbial communities' biodiversity. Moreover, different advanced techniques are presented that could shed light on the physiology of key community players, as well as specific applications of MCE in the WEFW nexus.

2. Monocultures vs microbial communities

MCE refers to the use of microbial consortia to advance highly complex tasks that are challenging to conduct with monocultures. In the biotechnological field, classic production of fine chemicals and food, such as amino acids, organic acids, antibiotics and enzymes is almost exclusively conducted in sterile cultivations, using primarily monocultures. On the other hand, microbial communities in natural ecosystems are characterized by multispecies interactions. In fact, assessing microbial diversity and the specific role of each species within complex environments is a difficult task since a fraction of the biotic phase is considered to be unculturable, i.e. typical laboratory conditions do not fully represent the complex environment where indigenous microbial population tend to grow. Thus, possible advantages derived from the interactions among microorganisms present in microbial consortia are fatally lost. Generally, microbial community dynamics are characterized by species–species and species–environment interactions, including symbiotic associations (i.e. mutualistic, parasitic, or commensalistic), competition, and predation within species and strains (Gómez-Camacho et al., 2019). A useful approach to select key species consists in the application of external stressors (*selective pressure*), for example by means of bulk parameters, to multispecies microbiota for the selection to act on the targeted species, which can be of interest to degrade lignocellulosic biomass and agro-industrial wastes.

2.1. Available tools in the field of MCE

Microbial communities, in the past, were often treated as “*black boxes*”, where the composition, function and stability of the system were not fully analysed. Only some particular microbial genes, functions, or metabolic products were well-studied, while the majority of the physiology rested undescribed. Nowadays, the *systems ecology* paradigm tries to integrate all the information gathered even at different scales, by high-throughput techniques, following an integrative physiology-environment approach. Especially multi-omics (Table 1) can potentially allow a better understanding and design of microbial communities. The field of MCE is of particular interest, since different integrative physiology approaches, such as top-down and bottom-up can be adopted, in order to study and tune key features in microbial populations. Furthermore, there are other high-throughput techniques, which can help in the field of MCE. For example, the flow cytometry (FC) platform allows morphological differences between members of microbial communities to be resolved and clustered according to key microbial groups, using (electro)optic properties (scattering) at the single-cell level (Silva et al., 2012). In addition, the use of dyes in FC measurements allows additional physiological information to be collected, especially if staining-steps are rationalized in MCE studies (Gómez-Camacho et al., 2018).

2.2 Higher-order interactions in MCE: Interspecies Electron Transfer, Quorum Sensing and Biofilms formation

The typical classification of cellular metabolism based on energy sources considers mainly phototrophic or chemotrophic organisms, the latter divided into chemoorganotrophic or chemolithotroph. However, recent studies have shown that some microbial communities exhibit complex mixotrophic mechanisms, where interspecies electron transfer (IET) plays a fundamental role, especially in shaping syntrophic relations. In particular,

two mechanisms are studied: Shuttled Interspecies Electron Transfer (SIET), which uses chemical shuttle molecules as electron carriers (e.g. H₂ or formate) and Direct Interspecies Electron Transfer (DIET), which relies on cytochromes, conductive pili or aggregates formation to take place (Gómez-Camacho and Ruggeri, 2018).

Table 1. Available omics tools for MCE

Omic tool & Information	Examples in the WEFW nexus
(Meta)genomics	<u>Cheese production and milk fermentation:</u>
Sequences from DNA (overall genes present)	Effect of influencing factors (salinity, pH, Temperature, moisture, cow feeding) on key microbiota players (Lactobacilli, Lactococci, Galactomyces, Aspergillus) as well as microbial spoilage (<i>C. butyricum</i> , <i>Eschericchi spp.</i>) (Yeluri et al., 2018)
	<u>Wine production (vineyard and winery):</u>
	Characterization of microorganisms in different sources along the production stages (Vineyard-soil, vineyard leaves, vineyard grapes), in particular fermenting bacteria (Firmicutes), spoilage bacteria (Acidobacteria, Proteobacteria) and fermenting fungi (<i>Saccaromyces</i> , <i>Hanseniaspora</i>) (Bisson, et al., 2018)
Transcriptomics	<u>Isopropanol production by <i>Clostridium beijerinckii</i>:</u>
Expressed genes (messenger and non-coding RNAs)	Regulation of clusters of genes (8 clusters, 953 genes) at different growth stages, useful to identify glycolysis clusters and carboxylic acids and solvents production shifts (up- and down-regulations) (Máté De Gérando et al., 2018).
Proteomics	<u>Functional metaproteome of contaminated soil and groundwaters</u>
Overall expressed proteins (microbial functionality)	Identification of functional groups (chaperones, protein synthesis, metabolism, degradation) in chlorobenzene contaminated soils and groundwaters, linked to the certain microbial genera (<i>Rubrivivax</i> , <i>Rhodoferrax</i> , <i>Ralstonia</i> , <i>Geobacter</i>) (Benndorf et al., 2007).
Metabolomics	<u>Anaerobic ammonium oxidation (Anammox) consortia:</u>
Metabolic products and end-products	Roles of acyl-homoserine lactones (AHL) family, in particular: 3OC6-HSL (N-(3-oxohexanoyl)-DL-homoserine lactone), C6-HSL(N-hexanoyl-DL-homoserine lactone), and C8-HSL (N-octanoyl-DL-homoserine lactone) controlling the electron transport carriers, 3OC6-HSL also in the regulation of metabolism and bacterial growth. The hypothesized mechanisms include amino acids production (alanina, valina, and glutamic acid) and selective regulation of aspartame and leucine to affect extracellular proteins, influencing aggregation (Tang et al., 2018).

On the other hand, many bacteria are believed to regulate and/or coordinate their social activities through cell-cell communication mechanisms, commonly termed *quorum sensing* (QS), mediated through autoinducer molecules. These coordinated behaviours include symbiosis, sporulation, germination, formation of fruiting bodies, bioluminescence, bacteriocin production, genetic competence, programmed cell death, virulence and biofilm formation (Li and Tian, 2012). Although several QS systems are known, the two most thoroughly described systems are the acyl-homoserine lactone (AHL) systems of many Gram-negative species and the peptide-based signalling systems of many Gram-positive species that involve the production of small linear or cyclic peptides (Parsek and Greenberg, 2005).

Microbial aggregations, in the form of biofilms, are composed of two main components: the biotic phase (microbial cells) and extracellular polymeric substances (EPS). Biofilms are characterized by enhanced resistance to chemical, physical and biological stresses, for this reason, they are able to colonize artificial or natural surfaces, of inorganic and organic nature. Biofilms found in many natural environments are frequently mixed species; at present, there is a growing interest in the importance of IET and QS in shaping multispecies communities. The presence of signal molecules has been observed and described in a few number of natural systems and considering that the signal could be altered by the natural environment, probably the influence of QS signals is confined to small areas containing clusters of cells (Decho et al., 2010). Nevertheless, little is known about how QS and IET interplay operate in nature and how to reproduce these signalling mechanisms in controlled bioreactor environments. A practical example, where the aforementioned phenomena have been studied is presented by (Tang et al., 2018). In this study, a metabolomics analysis conducted on an anammox consortium (Table 1) revealed the existence of AHL-based QS systems, which affected electron transport mechanisms (increasing NAD levels) and aggregation by promoting the formation of larger microbial flocs.

3. The Water-Energy-Food-Waste (WEFW) nexus

3.1 Food and Waste

The anthropocentric perception of production processes mainly considers input flows of materials and energy that are transformed into a specific target product, leading also to the production of by-products, waste and pollution. However, in biotic systems, chemoorganotrophic organisms obtain energy by extracting electrons from C-sources that is successively passed through different red-ox cycles, supported by enzymes under different electrochemical potentials (i.e. ferredoxins, flavodoxins, rubredoxins, NAD, FAD) until finally fixed in terminal electron acceptor (TEA) molecules. Depending on the degree of reduction C-molecules and the TEAs, a determined microorganism might utilize the metabolic products from another member as C-source. Classical fermented foods include, for example, yoghurt, cheese, kefir, wine, bread, beer, tempeh, vinegar, among many others. Other compounds of interest in the food industry include nutraceuticals and antioxidants. For example, carotenoids can be produced by co-culturing *Rhodotorula glutinis* and *Debaryomyces castellii*, exploiting the

polysaccharides hydrolytic capacity of *D. castellii*, and the carotenoids synthesis capacity of *Rh. Glutinis* out of the released sugars (Buzzini, 2001).

Certain waste and refuses streams from the food industry contain residual reduced C-sources, which can be exploited in additional biotechnological processes. For example, the production of polyhydroxyalkanoates (PHAs) via mixed microbial cultures under non-sterile conditions. PHA production via microbial communities is increasingly integrated into process technologies for the biological management of organic waste residuals and wastewaters such as fermented molasses, residues of paper production, olive mill effluents and several domestic and industrial wastewaters. This approach uses MCE and ecological selection principles, where microorganisms able to accumulate PHA are selected by the application of such operational conditions forced on the biological system. Therefore, the objective is to engineer the ecosystem, rather than the strains, adopting the methodology of environmental biotechnology in the field of industrial fermentations. Using engineered mixed cultures on different feedstock, a broad range of PHA compositions can be produced, with monomer units such as 3-hydroxyvalerate (3HV), 3-hydroxy-2-methylbutyrate (3H2MB), 3-hydroxy-2-methylvalerate (3H2MV), probably due to the fact that mixed cultures are able to employ a broader range of PHA production pathways. In fact, efficient PHA-storing organisms have been enriched in these systems through the application of feast and famine (FF) conditions, i.e. periods with substrate (feast phase) and without substrate (famine phase) in an aerobic sequencing batch reactor (Oliveira et al., 2017). Moreover, some examples of co-cultures fermentation are present in the literature and reviewed in the work of (Mazzoli et al., 2014); as an example, by applying of soft-modelling techniques, the PHB yield of a co-culture of *Ralstonia eutropha* and *Lb. delbrueckii* was increased by 19.4% compared with a pure culture (*R. eutropha*).

3.2 Water treatment and recovery

Water availability regulates microbiological activity and can potentially shape the structure of microbial communities; the nature uses microbiomes to assure the quality of the water as drinking as well as in natural attenuation of environmental matrixes from the origin of the live in our Planet. Here a brief recall of the MCEs use in some anthropological processes is reported.

Wastewater treatment plants are important infrastructures that ensure safe water supply and help to achieve urban sustainability, they play a vital role in environmental protection of local waters. Activated sludges (AS) are engineered artificial microbial ecosystems with high microbial diversity, containing over 700 genera and thousands of taxonomic units at high concentration of biomass. The use of AS is the most popular biological wastewater treatment application worldwide, it has been used to treat various types of municipal and industrial wastewaters and continues to be employed for purposes of public health and environmental protection. AS require, however, a large expenditure of energy to furnish the oxygen for the microbial activity to degrade organic pollutants present in the water. Recently, another candidate application of MCE is the Microbial fuel cells technology (MFCs) to treat wastewater and simultaneously generate electrical energy. In MFCs, microbes degrade complex compounds present in wastewater into substrates thanks to some 'exoelectrogen' bacteria. These bacteria play central roles in electrochemical interactions with electrodes; they are capable of transferring electrons to or receiving them from extracellular located red-ox active materials. In MFCs, electroactive bacteria catalyse the oxidative conversion of organic compounds into electricity splitting the oxidative bioreactions at the anode, generating low potential electrons that travel via external circuits to the cathode where reduction reactions occur. In microelectrochemical systems (MESs), electroactive bacteria can also catalyse the reductive production of valued chemicals using electricity supplied from electrodes. It is known that microorganisms alter their gene expression profiles and metabolic pathways in response of changes in intracellular red-ox states, which are regulated, for example, by the NADH/NAD⁺ and quinol/quinone ratios. Tschirhart et al. (2017) have recently shown that fine-tuning of gene expression and physiological behaviours (e.g. motility and cell-to-cell communication) might be modulated by electronic signals from red-ox mediators. For instance, *Geobacter sulfurreducens* expresses different inner membrane cytochromes depending on the electrode potential (Levar et al., 2017).

Bioremediation is another important application of MCE as it has been shown that microbial consortia perform better in the degradation of certain pollutants in aqueous environments than single species. Bioremediation uses microbial activity to degrade toxic pollutants. It offers an alternative to conventional remediation approaches, having as advantages: low-cost, more friendly technology (compared to incineration), can be carried out in situ, is generally environmentally more acceptable and generates lower amounts of waste. As the degradation of the pollutant is often a multistep process, the different microbial species composing the MCE consortium could be cultured sequentially, following engineered degradation steps. In situ bioremediation offers a valuable alternative to ex situ remediation approaches, as the microorganisms responsible for the degradation of the pollutants could be "inserted" into the environment as occurs in bioaugmentation ensuring suitable temperatures, pH, nutrients, etc. A possible solution to control the spatial distribution of MCE affecting the biodegradation is the construction of porous alginate beads, containing the microbial consortium and carbon

sources and dispersing them in the contaminated soils or water bodies. The physical characteristics of the beads can be customized to the microbial consortium; pollutants are expected to be transported by diffusion to the degraders trapped inside the beads, hence great attention must be devoted to mass transfer phenomena, which limit the performance.

3.3 Energy

Energy carriers that can be produced by microbial communities can be in liquid or gas phase. The production of biofuels has been classified into four generations (Table 2). The most extended one is the second generation, using biotechnological processes for gas energy carriers. Especially, Dark Fermentation (DF) and Anaerobic Digestion (AD) of different food and organic waste, for the production of biohydrogen and biomethane, respectively. AD exploits complex microbial cultures, mainly composed by Archaea (Euryarchaeota) and Bacteria (Firmicutes, Proteobacteria, Bacteroidetes and Chloroflexi) microorganisms (Campanaro et al., 2016), where different biochemical cycles take place (i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis). On the other hand, DF can be seen as a truncated version where methanogenesis is not present. Hydrolysis and the fermentative stages (acidogenesis and acetogenesis) are possible due to the action of bacteria, while methanogens are the terminal players in the microbial trophic chain, responsible for methane production from specific C-sources: acetic acid (acetoclastic methanogenesis) and CO₂ (hydrogenotrophic methanogenesis). The combined production of bioH₂ and bioCH₄ can be achieved using two-stage anaerobic digestion (TSAD) systems, selecting specific microbial consortia. For instance, (Gómez-Camacho et al., 2019) tested the influence of operational external stressors (pH, mixing rate) as well as the kinetic selection (i.e. based on the different growth rates of the involved microorganisms) and achieved stable bioenergy production from both stages, at different hydraulic retention times. TSAD performed better than traditional one-stage AD (in terms of energy recovery), since the first stage served as biological pre-treatment, thus making the substrates more easily biodegradable by methanogens in the second stage.

Table 2. Classification of biofuels generation

Gen.	Description
I	Derived from food crops and compete for arable land used in agriculture. Traditional chemical and mechanical processing (grinding, milling, distillation, transesterification, among others), and well-established biotechnological processes.
II	Uses food and organic waste/residues, which do not compete for arable land. The high content of cellulose and lignin present in this type of feedstock requires pre-treatment steps (chemical, thermal, mechanical and/or biological) to improve the yields (e.g. AD and DF).
III	Biomass cultivation, particular algae. This generation targets enhanced carbon dioxide (CO ₂) fixation. Autotrophic microorganisms that present higher growth rates than plants are preferred, particularly algae with high lipids metabolism (e.g. for biodiesel production). Drawbacks: high energy expenditures for cultivation & down-stream processing /microbial contamination
IV	the <i>state-of-the-art</i> generation of biofuels, that includes the use of advanced biotechnology for biofuels productions. It encompasses modified microorganisms (GMO) and microbial community engineering (MCE) to target specific products or to exploit symbiotic associations to enhance carbon capture, improve yields and modify important features of microorganisms.

In the liquid phase, important solvents such as ethanol and butanol could also represent an alternative for fossil fuels. However, the competition for arable land has directed the research towards the utilization of non-food materials, using agricultural waste with high lignocellulose content. Lignocellulosic biomass cell walls mainly consist of sugar-based polymers that are combined with lignin and a small fraction of extractives (cell wall chemicals mainly consisting of fats, fatty acids, terpenes, alcohols, phenols, resin acids, waxes, etc). In this respect, (Ransom-Jones et al., 2017) studied landfill cellulolytic communities in order to identify hydrolytic enzymes for biomass conversion. Their results suggest that Firmicutes, Spirochaetes, and Fibrobacteres are the key phyla in play, detecting more than 8 000 carbohydrate-active enzymes that encompass at least three different mechanisms for cellulose decomposition. Different engineered microbial co-cultures systems for the production of biofuels (e.g. ethanol, butanol, palmitic acid, oleic acid, muconic acid) have been reviewed by (Jiang et al., 2019) from the perspective of enhancing syntrophic relationships among pair of microorganisms, while (Du et al., 2015) achieved ethanol titers of c. 2.59 g/L from a cellulose concentration of 5 g/L at 55 °C by enriching a natural consortium (Firmicutes and Proteobacteria) with *Pseudoxanthomonas taiwanensis*.

4. Conclusions

The crucial role of microbial diversity in the challenges of the WEFW nexus cannot be disregarded. It is clear that understanding how microbial communities manage resources can allow improving current production and consumption patterns, aiming at developing comprehensive strategies for the next-generation bioprocesses. In this respect, several features of microbial communities composed by different strains and species must be studied in detail, such as metabolic plasticity, interspecies electron transfer and quorum sensing, whereas the development of advanced tools, including capabilities in multi-omics and high-throughput methods, could shed light on microbial ecosystems functioning and specific role and activity of community members.

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