

### *Chapter 3*

## **Lessons of the impact of genetically engineered micro-organisms on natural ecosystems like soil**

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*This chapter briefly describes some examples of past and present (next to potential novel) applications of genetically modified micro-organisms to soil, stressing the importance of analysing the putative impacts of such applications to the life support functions (LSF) of a living soil at three levels: i) functioning for soil fertility; ii) functioning for pathogen suppressiveness; iii) functioning for the provision of clean drinking water. To understand the impact of such genetically modified micro-organism applications on the soil, it is important to deepen our understanding of the microbial communities that are responsible for the key LSF of that soil. Moreover, we need to understand how these might be affected mechanistically. It is, therefore, important to further develop databases that contain extensive data on the microbial communities in the soil systems under study. This chapter advocates the application of the currently available powerful methods, which enable the dissection of soil microbial systems into their individual components. Finally, the chapter proposes the definition of a normal operating range (NOR) to fit the dataset obtained into a framework which is quantifiable and may serve to support decision making.*

## Introduction

### *Use of genetically modified micro-organisms*

Over the last 20 years, several genetically modified micro-organisms (GMMs) have been and are still being developed for use in agricultural and other (e.g. waste treatment) settings. An early approach to application in agriculture, incited in several Chinese research institutes, consisted of the use of a genetically modified *Alcaligenes faecalis* strain A1501. This organism, now renamed *Pseudomonas stutzeri*, had been modified to constitutively fix atmospheric nitrogen in the rhizosphere of rice, even in the presence of reduced nitrogen. The reduced nitrogen (in the form of ammonium) would otherwise repress the nitrogen fixation system (Lin et al., 2000). Following its development, the organism was used, at a large scale, in the rice-growing areas of the People's Republic of China, and slightly consistent increases of crop yields (5-10%), as measured by plant biomass and grain yield, have been reported (Lin, personal communication). Moreover, the applications did not exert any observable deleterious effects on the (agricultural) environment. Parallel microcosm studies with the strain performed in the Netherlands did not provide any evidence for measurable effects, neither on the colony forming unit (CFU) counts of indigenous bacteria nor on the PCR-DGGE profiles representing the community structures of total bacteria present in the system (Lin et al., 2000). The only discernible effect found was the one resulting from the (ephemeral) presence of the inoculant strain, which was, for instance, selectable on the basis of its great capacity to quickly utilise lactate.

Another application includes the now famous long-standing application of the GMM *Agrobacterium radiobacter* strain K1026, which was modified from the biocontrol agent *A. radiobacter* strain K84 to combat the plant pathogen *A. tumefaciens* (causing crown gall disease) in soil (Ryder and Jones, 1991). The modification was intended to block the transfer of toxin-resistance genes to cells of *A. tumefaciens*, which would turn these insensitive to the control. *A. radiobacter* strain K84 contains a plasmid, termed pAgK84, which encodes the anti-pathogen (toxin) compound agrocin 84, next to the gene conferring intrinsic resistance to this toxin. It also contains another plasmid, pNOC, which codes for nopaline uptake and catabolism. Thus, strain K84 competes with the pathogen at gall sites for nutrients (opines), killing the pathogen by producing agrocin 84. Strain K84 turned out to be an efficient coloniser of plant roots and wound sites, providing protection after application. However, the possible transfer of the agrocin plasmid to cells of the pathogen, brought about as a result of transfer functions carried on the pNOC plasmid, might result in pathogenic strains becoming resistant to agrocin 84 and hence a breakdown of the control. In order to avoid this potential breakdown, the transfer (*Tra*) region of pNOC was deleted by genetic modification, to produce the transfer-minus derivative strain of K84 termed *A. radiobacter* K1026. With great success, strain K1026 is now commonly used in many *A. tumefaciens* control strategies. It represents the first GMM approved for release into the environment (originally under Australian regulation). It is regarded as being safe for humans, animals, plants and the environment, being, except for the deletion of part of its genome (including the plasmid genomes), identical to the naturally occurring counterpart.

Chapter 1 provides an account of other promising applications of GMMs. The future for such and other environmental applications of GMMs thus appears to be bright. In most cases, the respective GMMs are designed for specific tasks in the environment, and hence they will inevitably exert the effects related to their intended tasks whenever they enter the environment.

### ***The potential (adverse) impact of genetically modified micro-organisms on ecosystems***

Notwithstanding the ample possibilities for a successful use of GMMs in the environment, there are still those that question the potential impact of the unintended effects that may be caused by the release of GMMs. To exercise this issue, one could consider the following: GMMs may theoretically impact their recipient environment by:

1. chemical (abiotic) modification of the environment
2. outcompeting, antagonising or cross-feeding the local microbiota, thereby changing their population structure
3. exerting effects on local organisms, such as plants, and/or
4. spreading their (inserted) genes by horizontal gene transfer (HGT).

Several of the above impacts are well measurable, whereas others may be more difficult to discern. For instance, a range of methods has been developed that allows an optimised detection of indigenous microbial communities (Kowalchuk et al., 2004; see below). Thus, the putative effects of GMMs on such communities can be determined in an elaborate manner. In addition, chemical changes of the environment are often also well measurable. HGT from the GMMs into the indigenous microbial community is also measurable, up to a particular level of resolution, using standard molecular screening techniques (Kowalchuk et al., 2003). Thus, the advanced methods that are currently available would facilitate a thorough assessment of the potential impact of GMMs. However, against which background should systems that are potentially impacted by GMMs be tested or compared?

### ***The need to define normality in target ecosystems like soil***

It is common knowledge that the chemistry of the natural environment, and the natural microbial and other communities that inhabit it, are often prone to fluctuations in response to the natural or anthropogenic influences that impinge on it. Hence, it is important to establish, in any ecosystem, what is supposed to be “normal” and what goes beyond “normality” (defining, with respect to the aforementioned parameters, a baseline or normal operating range, NOR). This goes beyond the type of effect that is expected to be exerted by a GMM. Then, the magnitude and duration of any effect of a GMM should be weighed against the amplitude of variation offered by the NOR. This chapter will discuss how a soil NOR can be determined and to what extent it may be useful as a baseline to weigh the potential impacts of GMMs against. But first the chapter will examine the difficulties posed for analyses of living soil systems, the methods that have been developed to overcome these and the key microbial functions of soil.

### **The great plate count anomaly and methods that can overcome it**

It is well known that a majority of the micro-organisms of soil does not easily grow on plates. This phenomenon has been coined the Great Plate Count Anomaly (GPCA; Staley and Konopka, 1985), and it can – for bacteria – amount to 99% of the total microbiota (Staley and Konopka, 1985; Ward et al., 1995). The GPCA impedes the easy and thorough understanding of the structure of soil microbial communities on the basis of traditional cultivation-based methods, simply because a majority of micro-organisms is unculturable and hence escapes functional analyses. The soil DNA- and RNA-based methods developed in the last two decades have provided a great thrust to man’s

understanding of soil microbial communities, as these provide snapshot-type descriptive information sets. Indeed, a large suite of advanced methods is currently available for the assessment of the microbial community structure and diversity in soils (Kowalchuk et al., 2004; Van Elsas et al., 2007). Moreover, more recent strategies to: *i*) apply DNA micro-arrays containing suites of probes that can report on the phylogenetic and functional status of soil communities; and *ii*) apply direct pyrosequencing to soil DNA samples, offer great potential to foster our understanding of the composition and functioning of the microbial communities (DeSantis et al., 2007; Roesch et al., 2007; Van Elsas and Boersma, 2011). Other methods that focus on microbial functions (e.g. based on the Biolog system) enable a view of functional diversity, without specifically assessing the underlying micro-organisms.

In the light of such and other recent methodological developments, one can safely state that an era has commenced, in which, for the first time in history, a more or less complete inventory of the community structures and diversities of the microbiota of soils can be made. The following section discusses the importance of soils for life on planet Earth, then examines to what extent this enormous methodological capability can assist in the quest to define the “normality” of soil and its functioning, in the light of the current and future applications of GMMs.

### **The soil ecosystem – its natural (normal) status, functioning and resilience**

As GMM applications in (agricultural) soil will undoubtedly be important, a special focus is placed here on the intricacies of the agricultural soil environment, in particular its quality and health status. This soil status is important as the following indispensable functions are supported:

1. the availability of plant nutrients (soil fertility)
2. the suppression of soil-borne plant diseases (see Chapter 2; Kennedy and Smith, 1995)
3. the cleaning function of soil, e.g. for the provision of clean drinking water (by filtering and biodegradation).

These three functions are known as the life support functions (LSF) of soil. They are very tightly linked to, and dependent on, crucial constituents of the soil microbial community. This section will briefly touch upon the first two functionalities.

#### ***Nutrient cycling function of soil***

Soils are responsible for a large part of the nutrient cycling processes (i.e. the cycling of different forms of carbon, nitrogen, phosphorus and sulfur) that drive ecosystem functioning on Earth. As examples, key steps in the nitrogen cycle such as nitrogen fixation, ammonia oxidation and denitrification, are carried out by micro-organisms that inhabit soils. In particular, ammonia oxidation and denitrification in soil determine what chemical forms of nitrogen, ammonium, nitrite or nitrate, will be available in (ammonium), or are flushed out (nitrate) of soil. Both processes are driven by several microbial groups, with the connotation that ammonia oxidation (carried by a few groups mainly among the beta-Proteobacteria and archaeae) is less broadly spread than denitrification (carried by many groups across the bacterial domain).

### ***Suppressiveness of plant disease***

The “health” of a soil can be defined in terms of its microbiological capacity to counteract (suppress) the activity of plant pathogens (see Chapter 2). This suppressiveness can conceptually be divided in “general” versus “specific” suppressiveness. General suppressiveness is defined as being caused by unspecified activities of a myriad of organisms (e.g. resultant from competition for essential nutrients with pathogens), whereas specific suppressiveness is related to a specific activity, e.g. antagonism, exerted by defined organisms.

Specific suppressiveness is classically best illustrated by the causal relationship between the decline of Take-all disease in wheat by consecutive wheat monocropping and the concomitant increase of fluorescent pseudomonads that produce the antifungal antibiotic 2,4-diacetyl phloroglucinol (Raaijmakers and Weller, 1998). Here, the GPCA would seem to impede a thorough assessment of the specific or even general disease-suppressive properties of the system, although Mendes et al. (2011) have recently pointed to a molecularly-based assessment of suppressiveness. In particular, the ecology of the key microbial interactions that take place in the soil environment and strategies to direct these, need scrutiny. It has been hypothesized that the level of interactiveness in a soil system is related to the stability of function, in this case suppressiveness.

### ***Resilience of soil***

It has often been postulated that the quality or health status of a soil not only relates to the soil’s functionality or disease suppression, but to its resilience in the face of stress as well, i.e. its capacity to return to the original status following such a stressful situation. In particular, disturbance of a soil microbial system may affect soil functioning to an extent that correlates inversely with the functional redundancy present in the system (Kennedy and Smith, 1995). This section argues that a greater microbial diversity, in particular in terms of function, may relate to a higher level of resilience, resulting in a better buffered system in the face of stressors that emerge. For both soils that perform nutrient cycling LSF and soils involved in pathogen suppression, this may mean that a greater diversity of nutrient-cycling, antagonistic or competitive functions correlates with higher degrees of resilience. It is of prime importance that methods are developed and applied that allow for a definition of the operational amplitude of healthy soil function, including “normal” responses to perturbances, versus what exceeds this NOR.

## **The normal operating range of soil function as the grand descriptor of normality**

In the light of the plethora of functions exerted by a normal agricultural soil, normality can be defined as a status of the soil under which all relevant functions are within the limits set by the normal climatic and anthropogenic influences exerted on the soil. When different relevant parameters are measured, their combination into one overall parameter would establish an overall NOR of soil function (Pereira e Silva et al., 2013). Here, it can be strongly argued that the modern molecular techniques (Kowalchuk et al., 2004; Van Elsas and Boersma, 2011) are indispensable tools that allow an optimised definition of the soil NOR. However, even with the advent of these advanced soil monitoring methods, the nature of the soil microbiota, its dynamics, activities and interactions may remain enigmatic for a long time to come. This is because such an intricate understanding requires the application of the tools at scales which are at the moment not realistic. In order to truly understand the normal fluctuations in soil, including those that result from agronomical measures, it is important that large databases



are created that allow the storage of large data sets, including those obtained by molecular tools as well as the so-called metadata (data that describe the key parameters of soil, i.e. soil pH, organic matter content, chemical status and textural type). The resulting description of the dynamic soil status will then provide the background against which out-of-range situations are compared (Bruinsma et al., 2003; Kowalchuk et al., 2003). Furthermore, important and sensitive indicators of soil processes should be selected. Given the presence of multiple functions in soil ecosystems, such a framework will rely on a range of soil attributes that, when considered together, will provide an estimation of the quality of soil in terms of its biological function (Villamil et al., 2007; Romaniuk et al., 2011).

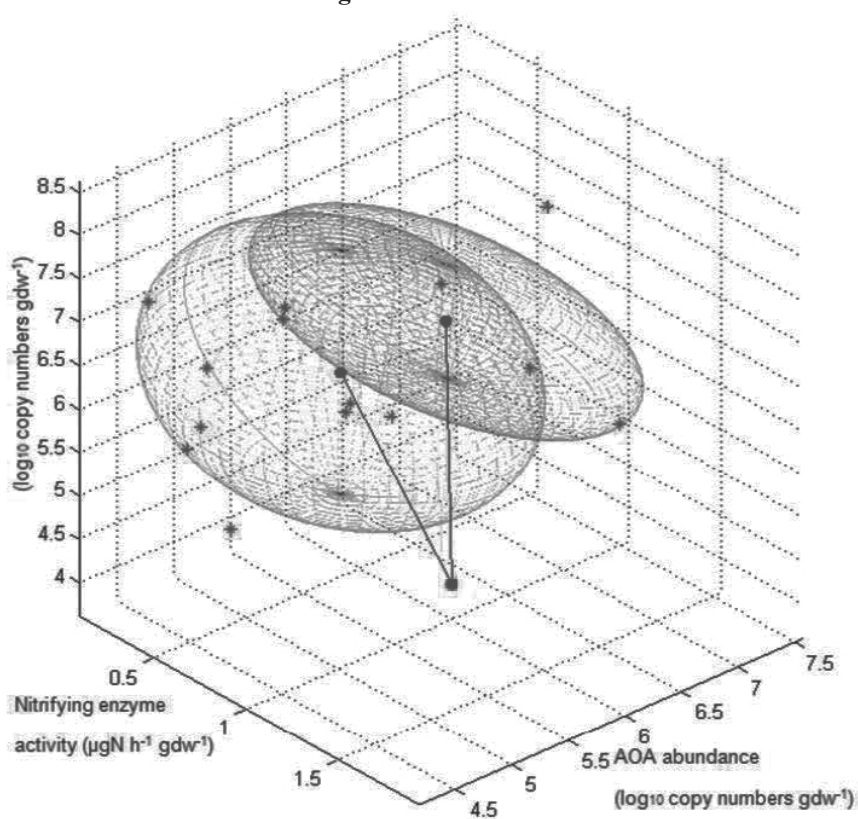
So, how can an NOR be established for soils? Looking to other parts of science where the NOR concept has been used, such as geochemical science (Wang et al., 2010), may help. In molecular ecology (De Boer et al., 2011), and recently in microbial ecology (Inceoglu et al., 2011; Pereira e Silva et al., 2011, 2012; Rutgers et al., 2009), the concept has been proposed, but so far no appropriate method or tool has been developed that satisfactorily defines the NOR of soils. To allow an assessment of the impact of practices such as the release of GMMs, the goal should be that key parameters quantify impacts for direct comparative purposes (Anderson, 2003).

A mathematics-based approach to defining the NOR is feasible. Let us consider the NOR as an ellipsoid in a space of  $n$  dimensions, where  $n$  is the number of parameters measured in a single system, and its borders represent the NOR. Such borders might be defined as the 95% confidence area of undisturbed states (Figure 3.1). They might also encompass all the data, so be defined by the extreme values that are still felt to be includable in normality. The distance between a particular state of the soil and the centre of the NOR will represent a quantitative measure that summarises the state of the soil, defined by Kersting (1984) as the “normalised ecosystem strain” (NES). Then, the strength of the “stress”, or how much a soil is outside the NOR, can be determined by the distance between the “stressed” soil and the border of the ellipsoid. When the soil is in an undisturbed state, all combinations of the parameters fall within the NOR, giving an NES value that is smaller than one unit. Values exceeding 1 would indicate that the system is under “stress”. The decision, however, whether a deviation of a soil from the NOR is “adverse” or not should be made by an educated guess with respect to the level of potential harm to the system. Ultimately, this would be a decision which is to be left to decision makers (Smit et al., 2012). The qualification whether a deviation represents harm or ecological hazard will depend on the use of the soil, e.g. for cultivation in agriculture or for nature development. It can only be done on the basis of the functions of that specific soil under evaluation (Rutgers et al., 2009).

Recently, an extensive study on particular soil parameters across Dutch soils was performed, over three consecutive years (Semenov et al., 2014). The study aimed to distinguish key soil parameters that could play an important role in the proper establishment of an NOR for soil function. In total, 22 measurable parameters were selected to define the NOR, including soil pH, organic matter, level of nitrate, abundance of bacteria, archaea, fungi, ammonium oxidizers, nitrogen fixers and denitrifiers. Moreover, nitrification and denitrification potentials were measured. In the work, the distance observed between a “stressed” soil and the NOR border, as based on nitrification-related parameters (activities, abundance and diversities), was much higher than the corresponding value between the NOR based on other relevant parameters (e.g. soil pH, OM, archaeal and fungal abundances and diversities; Pereira e Silva et al., 2013). The NES value was also higher when compared to the NOR based on more

redundant proxies (e.g. denitrification potential and abundance of total bacteria and denitrifiers). This supported the posit that by focusing on sensitive parameters such as those describing nitrification (taking the abundance, structure and function of ammonia oxidizers as parameters), a sound NOR of soil functioning is achieved. Consequently, the chances of distinguishing stressed soils (measurements outside the NOR) are likely higher when so-called sensitive parameters are considered than when randomly selected parameters are tested (Figure 3.1). Based on the above, a classification of potential biological parameters can be provided (Pereira e Silva et al., 2013), where nitrification-related measurements were top-ranked in relation to other (more redundant) measurements. However, it is noteworthy that these parameters were more sensitive in the sandy soils, supporting the idea that NORs should be built taking into consideration the type of soil under scrutiny.

Figure 3.1. **Representative example of a normal operating range of soils showing 3 of the 22 dimensions**



*Notes:* The ellipsoid 1 characterises the normal operating range for agricultural soil under tillage while ungrazed grassland is represented by the ellipsoid 2. The ellipsoids represent the borders of the NOR for three indicators (nitrifying enzyme activity and abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB)). Crosses (red) are observed values which characterise the normal operating range. The line is the distance between the centre of the normal operating range (dot (blue)) and the state of the selected soil (faint dot (green)). It is important to mention that the distance that reflects how much the selected soil (faint dot (green)) is outside the normal operating range is the distance between the faint dot (green) and the border of the ellipsoid, not the centre. Two ellipsoids are different in volume due to higher amplitudes of variation observed in the above-mentioned indicators for more disturbed soils (agricultural) compared to the grassland one.

*Source:* Pereira e Silva et al. (2013), “Microbe-mediated processes as indicators to establish the normal operating range of soil functioning”, *Soil Biology & Biochemistry*, No. 57, pp. 995-1002.

## Outlook

This chapter examined some key examples of the past and present (next to potential novel) applications of GMMs to soil and questioned the putative impacts of such applications to soil LSFs at three levels:

1. functioning for soil fertility
2. functioning for pathogen suppressiveness
3. functioning for drinking water provision.

To understand the impact of such GMM applications, it is important to deepen our understanding of the microbial communities that are responsible for the soil LSF and how these might be impacted. It is, therefore, important to focus on the further development of databases that contain extensive data on the microbial communities in the soil systems under study and are generated with the currently available powerful methods that enable the dissection of soil microbial systems into their individual components (Kowalchuk et al., 2004; Van Elsas and Boersma, 2011). Such databases should contain, next to the data on microbial communities, metadata that describe the local conditions. The microbiota-related dataset should be established at both the functional and phylogenetic levels, with an additional focus on the spatial and temporal relationships between the individual organisms and functions analysed.

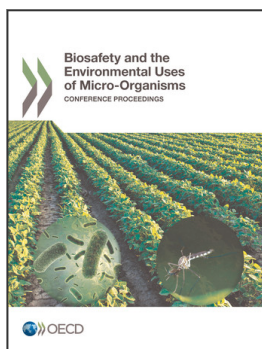
This chapter presented a strategy that might define an NOR of soil function, which includes the ranges of variation incurred by the different parameters that are deemed important as descriptors of the soil NOR. In this perspective, nitrogen cycling was taken as a key asset that primarily might define the soil NOR. However, it is important to bear in mind that such a proposal to pinpoint a particular process as being more important than other processes is somewhat arbitrary, and is thus open to discussion. Another issue raised here is that soil NORs may be implemented per soil type, assuming that particular key soil processes run in similar ways per soil type, which is supported by some recent literature (Pereira e Silva et al., 2011; 2012). However, in this still-developing area, the novel datasets are expected to allow further fine-tuning the current, assumption-based inferences about how soil NORs can be best established. Finally, the datasets that will allow, for the first time in history, having a comprehensive overview of the complex soil microbiota, will need to be supported by powerful bioinformatics tools that enable a ready and fast ordination of the data. There is a problem (once denoted as the “informational or computational bubble”), as such tools are currently not available. Hence, investments in bioinformatics are dearly needed.



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