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Sense and nonsense of the secondary metabolites data requirements in the EU for beneficial microbial control agents

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ABSTRACT

Microbial control agents (MCAs) are promising for use in sustainable agriculture. Nevertheless, they face a major hurdle in the registration process of the European Union, one reason being unclear data requirements on metabolites. This review examines the EU regulatory perspective on metabolites of MCAs for plant protection and identifies some key issues and concerns. Current data requirements for secondary metabolites of micro-organisms are based on degradation products/metabolites of chemicals. We conclude that this has contributed strongly to the current confusion regarding how to best evaluate potential production of toxic substances by MCAs. We suggest that data requirements should be revised and/or need guidance fit for purpose, in order to give the EU-regulation for MCAs a stronger base in microbiological knowledge. We also suggest implementation of a basic hazard assessment. If this is passed without any concerns, the production of unknown, potentially toxic, substances does not need to be further investigated.

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Table 1
Different terms used to indicate biologically derived control agents.

Biological control agents include microorganisms, nematodes, predators and parasitoids.
Microbial control agents (MCAs) are biological control agents consisting of microorganisms (fungi, bacteria, viruses or protozoans).
Biological pesticides, also called biopesticides, include the microorganisms, semiochemicals, botanicals and essential oils.

1. Introduction

1.1. Scope

There is a growing public concern about the impact of chemical pesticides on human health and the environment. In Europe, the Sustainable Use of Pesticides Directive 2009/128/EC (EU, 2009a) advocates the use of integrated pest management (IPM) with preference being given to environmentally friendly, non-chemical methods of pest control (EU, 2009a). Biologically derived control agents have an important position among these alternative methods (Table 1). They entail the use of macro- and micro-organisms as well as insect and plant derived substances. In this review we focus on microbial control agents (MCAs) used in plant protection, but our view is also relevant for microbial biocides and the Biocidal Product Regulation.

1.2. What is the problem?

The introduction of MCAs into the European market for practical use in pest and disease control is a very slow and costly process. Several authors state that Europe is lagging behind other countries when it comes to implementation of microbial pest control in practice and that this is primarily due to the restrictive regulatory conditions (Balog et al., 2017; Frederiks and Wesseler, 2019; Kabaluk et al., 2010). Table 2 illustrates that EU lags significantly behind the USA, India and China when it comes to numbers of MCAs registered as plant protection products. One area which has been particularly problematic and caused delayed evaluations has been the potential production by MCAs of secondary metabolites.

1.3. Goals of this review

In order to improve the regulatory situation for MCAs in the EU,

Table 2

Number of approved products worldwide¹ based on representative genera of bacterial and fungal BCAs (based on Kabaluk et al., 2010). For details of the secondary metabolites produced by these and other MCAs see OECD (2018b).

MCA genus	Examples of secondary metabolites	CH	I	USA	URM	EU	SK	CA	SA	NZ	AU	K	AR
Fungi													
<i>Beauveria</i>	Beauvericin, Oosporein		12	3	2	2	2	2	3	2	0	2	2
<i>Metarhizium</i>	Destruxins		9	1	1	0	0	1	1	0	3	0	0
<i>Trichoderma</i>	Trichodermin		15	3	4	8	0	2	9	6	1	4	1
<i>Lecanicillium</i> (was <i>Verticillium</i>)	Destruxins Bassianolide, Cyclosporin		14	1	2	3	0	1	0	1	0	0	0
All other minor species			10	18	4	8	3	7	3	2	0	4	0
Total for fungi		22 ³	60	26	13	21	5	13	16	11	4	10	3
Bacteria													
<i>Bacillus thuringiensis</i>			6	11	6	6	14	7	6	11	13	8	6
<i>Bacillus</i>	Surfactin, Fengycin		0	12	6	1	12	7	5	2	1	0	2
<i>Pseudomonas</i>	2,3-deepoxy-2,3-didehydro-rhizoxin (DDR) ²		7	4	7	3	0	2	0	0	0	0	0
<i>Streptomyces</i>	Streptomycin, Thaxtomin A		0	2	2	1	2	2	0	0	0	0	0
All other minor species			0	2	4	2	1	2	1	3	1	0	0
Total for bacteria		270 ³	13	31	25	13	29	20	12	16	15	8	8
Grand total		292	73	57	38	34	34	33	28	27	19	18	11

¹ China (CH), India (I), USA, Ukraine, Russia and Moldova (URM), EU, South Korea (SK), Canada (CA), South Africa (SA), New Zealand (NZ), Australia (AU), Kenya (K), Argentina (AR).

² EFSA (EFSA, 2017); in *Pseudomonas chlororaphis*.

³ Total numbers of products provided, not numbers of species or strains.

there is clearly a need to understand the main ambiguities of the data requirements, and what has caused them. This review examines key drivers and makes recommendations to develop data requirements more fit for purpose.

In this paper, we:

- present an outline of the regulatory process of MCAs in Europe and the evolution of legislation and data requirements for MCAs;
- examine and summarise the functional roles of secondary metabolites of micro-organisms in ecosystems;
- identify flaws and inconsistencies in the current data requirements for metabolites;
- discuss the current risk assessment approach and suggest improvements;
- summarise our recommendations.

2. Evolution of data requirements from a historical perspective

In Europe, MCAs for plant protection fall under the same EU regulatory frameworks as the chemical plant protection products (Regulation (EC) No 1107/2009 (EU, 2009b)), although there are specific data requirements for the micro-organisms. The data requirements for MCAs were developed by the end of the 1990's and published in Commission Directive 2001/36/EC (EU, 2001). At the time of its inception, regulatory experts did not have much experience of MCAs, presumably because the biological control industry was still in its infancy. Below we highlight key issues that we find strongly influenced the evolution of the data requirements for microbial secondary metabolites (Fig. 1).

2.1. Fear is the driver for equating secondary metabolites of MCAs with the harmful products produced by mycotoxigenic fungi

We assume that, at the time of drafting the data requirements, there was a concern that commercial MCAs may be developed from harmful micro-organisms, such as mycotoxigenic fungi which are known to produce toxic secondary metabolites. Drawing comparisons between beneficial MCAs and harmful toxigenic micro-organisms is understandable since the micro-organisms may be phylogenetically related. Consequently, data requirements are general and broad enough to accommodate both current and future microbial products, including possibly toxigenic micro-organisms. Although this may seem logical, in

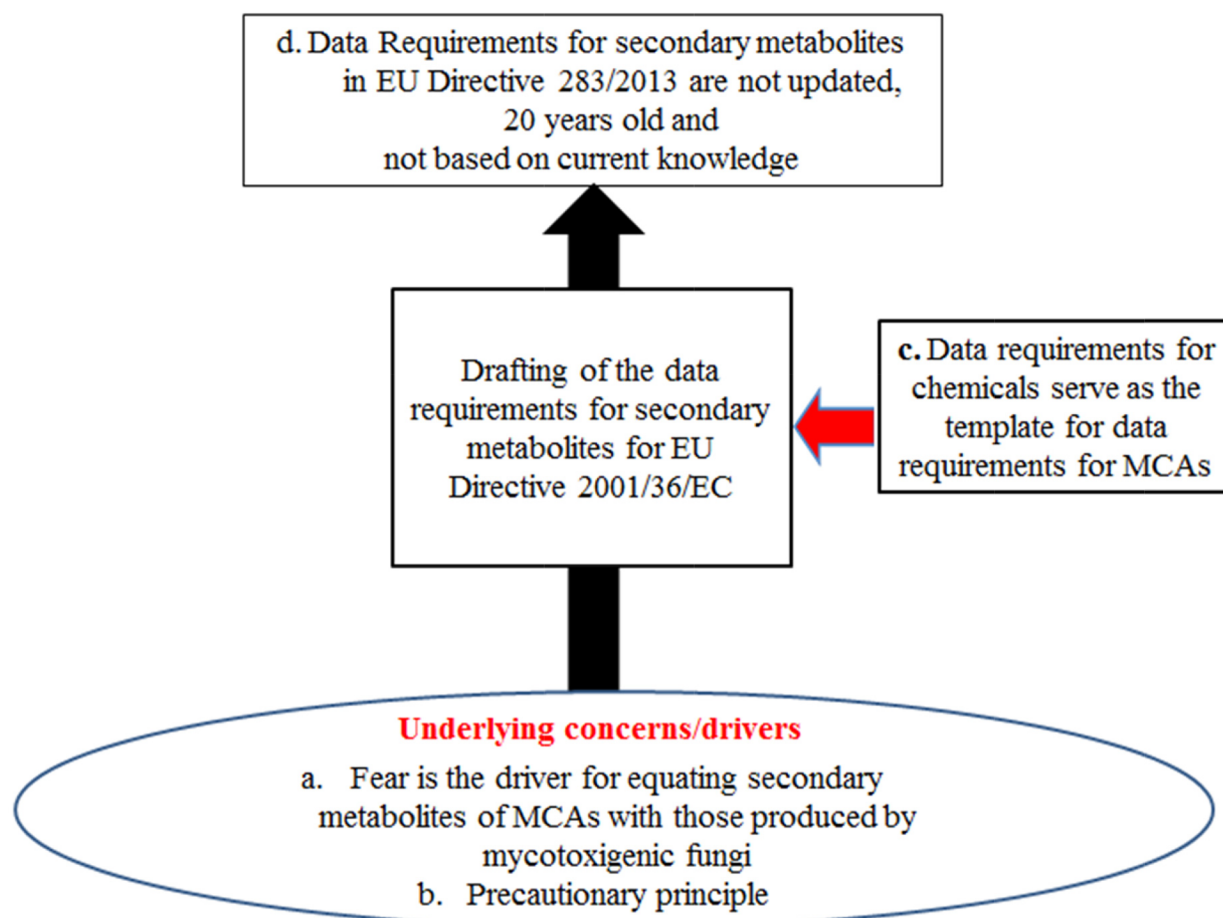


Fig. 1. Four factors (a–d) (representing sections 2.1–2.4) that influenced the text for secondary metabolites (SMs) in the EU data requirements (DRs) for microbial plant protection agents.

our opinion it is a hindrance. The text of the current data requirements is now fuelled by the evident risks posed by harmful fungi and bacteria, with fears based foremost on some well-known mycotoxins (e.g. aflatoxin, ochratoxin, fumonisins, trichothecenes, zearalenone) produced by toxigenic fungi such as *Aspergillus*, *Penicillium* and *Fusarium* species.

The fear that MCAs could pose a serious and unpredictable risk because of their potential ability to produce toxic metabolites remains to date (Deising et al., 2017). However, counter arguments have been presented (Koch et al., 2018; Lugtenberg, 2018). Secondary metabolites of MCAs are often very different from those produced by harmful micro-organisms even though they may involve the same biochemical pathways, demonstrating the genetic controls are quite different.

2.2. Precautionary principle

Regulatory experts often ask the question “can MCAs produce *unknown* secondary metabolites that might be harmful to humans and other non-target organisms?” This derives from the precautionary principle applied in the EU, which effectively states that an MCA cannot be approved, unless it is demonstrated to be safe. The question about unknown secondary metabolites is understandable but it ignores the natural roles of secondary metabolites for increasing the ecological fitness of micro-organisms in the environment. ‘Unknown’ is equaled with ‘unsafe’. This approach is one of the key problems in the risk assessment of secondary metabolites.

2.3. Data requirements for chemicals served as the template for the data requirements for MCAs

Risk assessment of MCAs is essential to reassure that the product poses no unacceptable risk to the general public or the environment and that the product is efficacious. The history of this regulatory process has been outlined in several reviews (e.g. (Cook, 1990; Lord, 2005; Ravensberg, 2011; Sundh and Goettel, 2013).

It is highly relevant in this perspective that under the previous Directive 91/414/EEC for plant protection products, data requirements were first developed for the chemical pesticides and that these formed the template for the data requirements for MCAs. Consequently, the data requirements for MCAs, including those about secondary metabolites, were strongly influenced by risk assessment criteria for chemicals. This was done as a matter of convenience since experience with microbial pesticide assessments was lacking, or at least not taken into consideration at that time.

2.4. Data requirements for MCAs are not based on current knowledge

Whereas the data requirements for chemicals were updated in the current Commission Regulations 283/2013 and 284/2013 (EU, 2013a,b), the data requirements for micro-organisms in these regulations were copied from the earlier directive of 2001. This was done in spite of the expansion of knowledge on MCAs and the experience gained by risk assessors and regulatory experts in the period between 2001 and

2013 on this topic. The missed opportunity to update the data requirements for MCAs and their secondary metabolites has meant that the applicants and risk assessors still have to comply with the data requirements of 2001.

Conclusion and recommendation (1)

The current data requirements for MCAs are outdated. We conclude that they were formulated with a fear that the well-known mycotoxins produced by toxigenic fungi are typical examples of secondary metabolites of micro-organisms. Additionally, the data requirements for chemicals, specifically those for their degradation and elimination products (i.e. 'metabolites'), were incorrectly copied to the data requirements concerning metabolites of micro-organisms. There is clearly a need to redraft the data requirements for secondary metabolites of MCAs to make them more pertinent to the properties of micro-organisms.

3. Knowledge on metabolites of MCAs relevant for data requirements for risk assessment

3.1. Natural roles of secondary metabolites in ecosystems

Being micro-organisms, MCAs produce a range of secondary metabolites which play a pivotal role in their development and ecology. The secondary metabolites can have a profound impact on other organisms

(Fig. 2) and include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, behavior-modifying chemicals (kairomones, pheromones), immune-modulating agents and plant growth promoters (Bérđy, 2005; Butt et al., 2016; Demain and Sanchez, 2009; Katz and Baltz, 2016; Netzker et al., 2015; Tyc et al., 2017). There are many secondary metabolites which remain to be identified and whose ecological role has not yet been established, which can perturb regulatory experts who might want to know the function of these compounds. For instance, the number of known bioactive metabolites produced by actinobacteria is over 10,000. The genus *Streptomyces* approximately accounts for 70–80% of this number (Bérđy, 2005).

3.2. Do secondary metabolites of MCAs persist and accumulate?

Micro-organisms and their secreted products, whether they be primary or secondary metabolites or other types of substances, will enter the pool of detrital organic matter which is gradually transformed and mineralised in the degradative food webs in both terrestrial and aquatic environments. Grazing invertebrates and protozoans play an important role as consumers of micro-organisms and, inadvertently, their metabolites in natural ecosystems (Scheepmaker and Butt, 2010). Besides natural decay due to edaphic and climatic factors there are many studies demonstrating degradation of microbial secondary metabolites whether they be by specific isolates or complex consortia (McDowall et al., 2009; Rastogi et al., 2015; Sato et al., 2012; Wright et al., 2014).

Some MCAs are applied to the plant foliage and form part of the epiphytic microbial community. These micro-organisms and their metabolites are also subject to degradation due to a range of biotic and

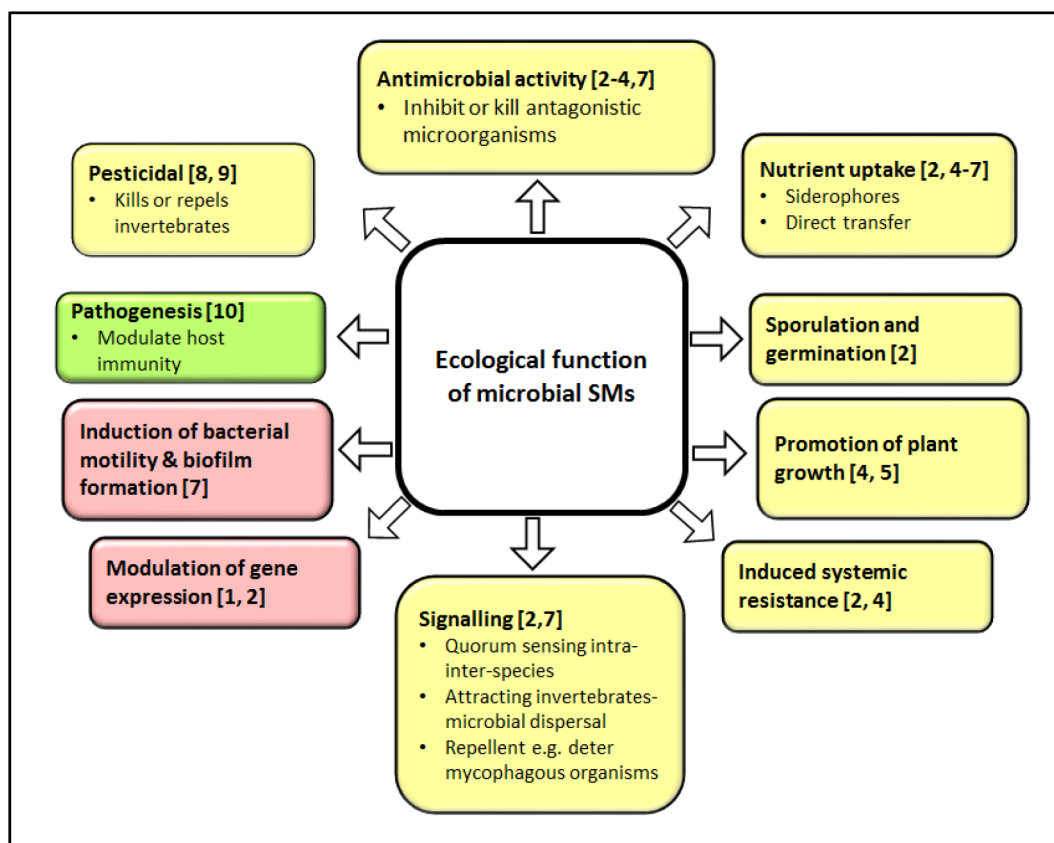


Fig. 2. Graphical summary of the functions of microbial secondary metabolites in nature. Yellow boxes – attributes shared by both fungi and bacteria, Green box -attributes more specific to entomopathogenic fungi (EPF), Pink boxes – attributes more specific to bacteria.1 (Humphris et al., 2002); 2 (Contreras-Cornejo et al., 2016); 3 (Arguelles-Arias et al., 2009); 4 (Liu et al., 2017); 5 (Behie et al., 2012); 6 (Raya-Díaz et al., 2017); 7 (Chowdhury et al., 2015); 8 (Cianco et al., 2016); 9 (Bogner et al., 2017); 10 (Butt et al., 2016).

abiotic factors, some of which overlap with those observed in the subsurface environment, such as grazing by invertebrates and protozoa and secondary metabolite degradation by plant associated micro-organisms (Sato et al., 2012; Wright et al., 2014). The plant canopy is a hostile environment for most micro-organisms since it is less buffered to climatic change compared to the soil. Fluctuations in temperature and humidity can have a profound impact on microbial development. UV light can oxidise and subsequently inactivate metabolites and kill the producers of those compounds (Fargues et al., 1996; Kaiser et al., 2019). Furthermore, rain can wash off the micro-organisms from leaf surfaces so they end up in the soil (Inglis et al., 1995; Inyang et al., 2000).

3.3. Quantifying secondary metabolites of MCAs

Mass production of MCAs is a highly controlled process. The resulting fermentation product can be easily checked for known toxins using chemical standards. Extraction and analytical techniques are however only available for a few well-known metabolites. In contrast, determining quantities of specific metabolites in the target host or environment (e.g. soil, plant) is a big challenge since the production of these metabolites is influenced by a set of highly dynamic environmental factors such as climate, microbiome, crop type etc. A rich mixture of disparate compounds is present in these environments and it is often difficult to trace their source. Some microbial metabolites may be very difficult to extract and purify because they are short-lived and/or present in small quantities. Furthermore, their production is multi-factorial and highly dependent on scale, making it difficult to predict when and where they should be measured. The problem is compounded by the possible production of similar metabolites by natural populations of microbes occupying the same niche as the applied MCA.

Data on quantities of secondary metabolites in the natural habitats of MCAs are very scarce (Hackl et al., 2015; Mudgal et al., 2013). Of the few compounds reported in a recent OECD report (OECD, 2018b), the quantities of secondary metabolites were in all cases low and could only

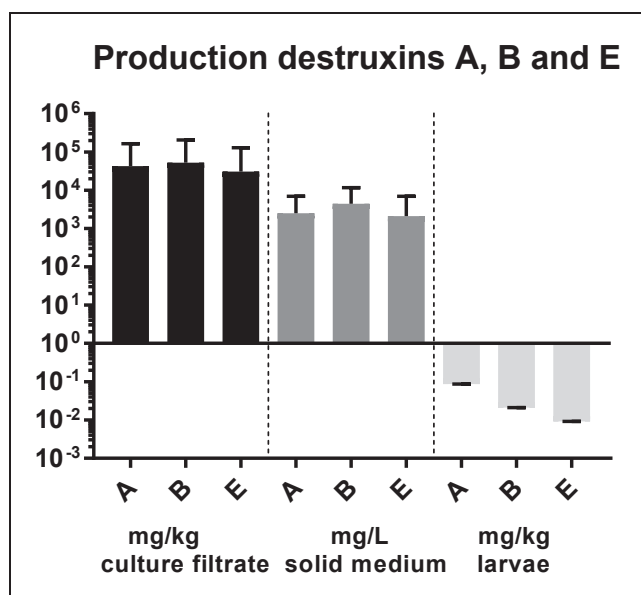


Fig. 3. Production of destruxins A, B and E by different strains of *Metarhizium anisopliae* in culture filtrate, solid medium and insect larvae. Data for culture filtrates and solid media derive from (Amiri-Besheli et al., 2000; Chen et al., 1999; Hsiao and Ko, 2001; Hu et al., 2006; Hutwimmer et al., 2010; Liu et al., 2000, 2007; Loutelier et al., 1996; Moon et al., 2008; Rao et al., 2006; Seger et al., 2006, 2004; Wahlman and Davidson, 1993; Wang et al., 2003, 2004, 2009). Data from larvae derive from (Amiri-Besheli et al., 2000; Skrobek et al., 2008; Suzuki et al., 1971).

be measured at high population densities. Furthermore, MCAs, like other micro-organisms, do not grow exponentially in the environment, in contrast to the situation in fermenters. Here, not only do they have access to unlimited nutrients but are also free of the competitors and predators of real ecosystems. To illustrate the point, the quantities of destruxin A, B and E produced by *Metarhizium* species in liquid fermenters is at least a million-fold more than that produced in an insect host (Fig. 3). It should be noted that these high concentrations are not representative of concentrations present in the final product. This is because the bulk of the secreted secondary metabolites are not harvested with the microbial propagule but left in the spent medium (Skrobek et al., 2008).

Conclusion and recommendation (2)

Secondary metabolites in natural ecosystems are not easy to quantify and consequently exposure data are usually missing. The quantities produced in the fermenter do not reflect the range or quantities produced *in situ* (on plant, in insect etc.) after application of the plant protection product. Measuring secondary metabolites *in situ* is therefore not a useful option, except perhaps if the MCA produces a known toxin.

3.4. Metabolites of micro-organisms and degradation products of chemicals are radically different

First of all, we need to understand what Regulation 1107/2009 means by ‘metabolite’. Point 32 of Article 3 in the regulation states: “any metabolite or a degradation product of an active substance, safener or synergist, formed either in organisms or in the environment”. This text could be purposefully broad and overarching to cover both chemical and microbial plant protection products but in reality, it fails to take into account the difference between degradation products of chemicals and metabolites of MCAs.

After application to crops, chemical pesticides will be broken down, by for example enzyme degradation, UV light, or transformation by metabolism in living organisms. Within the regulation the terms “metabolite”, “breakdown product” and “degradation product” are used interchangeably. Irrespective of the way of degradation or transformation of a chemical pesticide, the original compound will not be intact anymore.

In contrast to degradation products/metabolites of chemical pesticides, microbial secondary metabolites cannot be considered to be breakdown products of the original micro-organism. Micro-organisms though, produce primary metabolites (Fig. 4) which are required for the growth and maintenance of cellular functions and include amino acids, nucleotides, vitamins, solvents and organic acids. Secondary metabolites are products of secondary metabolism and are important in ecological and other activities of micro-organisms (see section ‘Natural roles of secondary metabolites in ecosystems’) and as such are an integral part of the organism’s survival strategy in the environment.

Micro-organisms can also grow in the tissue of plants. These plant endophytes (Fig. 4) represent many major taxonomic groups of micro-organisms, and include genera which form actives in commercial MCA-based products e.g. *Bacillus*, *Pseudomonas* and *Beauveria* (Card et al., 2015; Liu et al., 2017; Vidal and Jaber, 2015). These endophytic micro-organisms may exert biological control of herbivorous insects, and confer other benefits to the plant such as improved nutrient acquisition and tolerance to abiotic or biotic stress (Batta, 2013; Glassner et al., 2018; Kefi et al., 2015).

When drafting the data requirements for MCAs, the term metabolite was incorrectly copied from the chemical data requirements. This caused the problem that microbial metabolites were more or less treated with the same dread as degradation products/metabolites of chemical pesticides, although their origins and functions are entirely

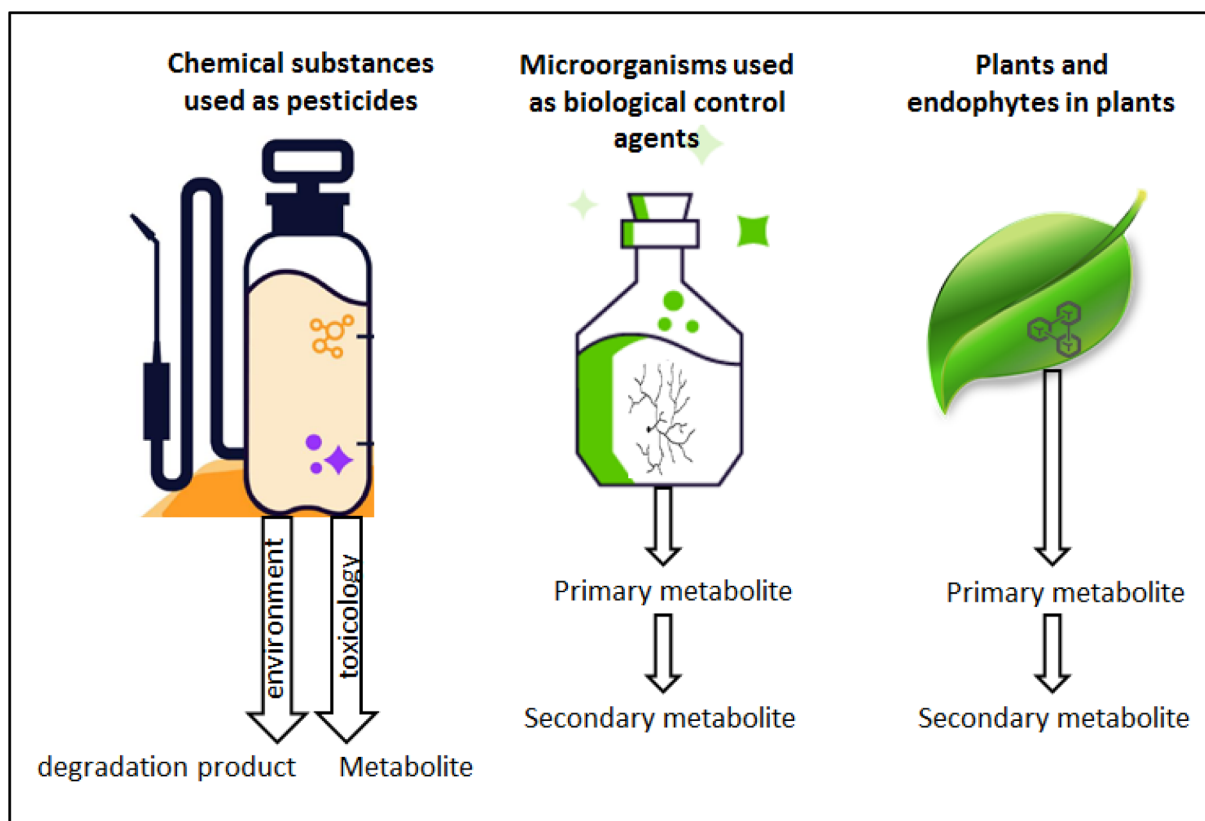


Fig. 4. Different possibilities of formation of ‘metabolites’ from chemicals, microorganisms (either MCAs or endophytes) and plants.

different. The use of the same term ‘metabolite’ for two very different kinds of substances significantly adds to the misunderstanding, but is unlikely to change because the term is firmly embedded in the scientific and risk assessment literature. Lucid, robust definitions are required which distinguish between metabolites and breakdown products from either microbial or chemical sources and the relevant sections in the regulation and data requirements should be rewritten accordingly. In addition, there is a need to increase the awareness among stakeholders, including the regulatory experts, of the differences between these two radically different types of substances.

Conclusion and recommendation (3)

MCAs and consequently their secondary metabolites are not known to accumulate in the environment because they are degraded within complex microbial communities/degradative food webs. Therefore, we suggest that not too much emphasis need to be placed on data requirements on the secondary metabolite degradation in the environment.

Metabolites and breakdown products from either microbial or chemical source are two radically different substances. Lucid, robust definitions are required which distinguish between these. The relevant sections in the regulation and data requirements should be rewritten to reflect this. There is also a need to increase the awareness among stakeholders of the differences between metabolites from microbials and those from chemicals.

4. Flaws in the current data requirements for metabolites

4.1. The term ‘relevant metabolite’ is ill defined

The definition in Article 3(32) of the Regulation 1107/2009 is: “A metabolite is deemed relevant if there is a reason to assume that it has intrinsic properties comparable to the parent substance in terms of its biological target activity, or that it poses a higher or comparable risk to organisms than the parent substance or that it has certain toxicological properties that are considered unacceptable. Such a metabolite is relevant for the overall approval decision or for the definition of risk mitigation measures”.

In this definition, the metabolite is compared with the ‘parent substance’. The definition is therefore exclusively referring to the toxicity of a chemical agent. Such a comparison is highly significant for toxicity evaluation of chemical actives since intermediates (‘metabolites’) in the degradation pathways of the compound may be equally or even more toxic than the parent compound. However, this definition of a ‘relevant metabolite’ of a chemical is not useful in the evaluation of secondary metabolites of MCAs, since it ignores the biological and ecological properties and roles of secondary metabolites produced by micro-organisms.

EU regulation 283/2013 contains the most recent publication of the data requirements for both the chemical and microbial active agents (parts A and B, respectively). Those for the microbial active agents though, were not changed since 2001. In part A for chemical substances certain environmental studies are requested for “metabolites, breakdown and reaction products” when “they account for more than 10% of the amount of active substance added at any time during the studies” (see Data requirement 7.1.2.1.2). Thus, degradation products accounting for

Table 3

Data requirements in Commission Regulation (EU) 283/2013 for micro-organisms requesting information on metabolites. In bold Data requirement 2.8 is specifically addressing secondary metabolites.

Section/Data requirement	Primary focus of data requirements	Indication of the metabolite in the text of data requirements
Introduction (viii)	Micro-organism	Toxins/metabolites
1.4.2 Identity and content of impurities, additives, contaminating micro-organisms	Micro-organism	Relevant metabolites
2.2.2 Mode of action	Micro-organism	A toxin with a residual effect; metabolites (especially toxins)
2.4 Development stages/life cycle of the micro-organism	Micro-organism	Metabolites, including toxins that are of concern for human health and/or the environment
2.5 Infectiveness, dispersal and colonization ability	Micro-organism	Relevant toxins
2.8 Information on the production of metabolites (especially toxins)	Secondary metabolite	Metabolites (especially toxins) with unacceptable effects on human health and/or the environment
4.2 Methods to determine and quantify residues (viable or non-viable)	Micro-organism	Relevant metabolites (especially toxins)
6.1 Persistence and likelihood of multiplication in or on crops, feeding stuffs or foodstuffs	Micro-organism	Relevant secondary metabolites (especially toxins)
6.2.1 Non-viable residues	Micro-organism	Metabolites, especially toxins
7 Fate and behaviour in the environment, introduction	Micro-organism	Relevant metabolites

Table 4

Information to be provided under Data requirement 2.8.

If other strains belonging to the same microbial species as the strain subject to the application are known to produce metabolites (especially toxins) with unacceptable effects on human health and/or the environment during or after application following information should be provided	
1	The nature and structure of this substance
2	Its presence inside or outside the cell and its stability
3	Its mode of action (including external and internal factors of the micro-organism necessary to action)
4	Its effect on humans, animals or other non-target species
5	The conditions under which the micro-organism produces the metabolite(s) (especially toxin(s)) must be described
6	Any available information on the mechanism by which the microorganisms regulate the production of the(se) metabolite(s).
7	Any available information on the influence of the produced metabolites on the micro-organism's mode of action.

more than 10% of the parent seem to be considered as 'relevant'. It is clear that the 10% of the active substance cut-off rule can only refer to the breakdown products of chemical substances since metabolites of micro-organisms cannot be expressed as a percentage of the micro-organisms. An attempt to define the relevant metabolite in part B for micro-organisms (without using the 10% rule) is given in point 1.4.2: "Relevant metabolites (i.e. if expected to be of concern to human health and/or the environment)....". Unfortunately, this definition fails to clarify how much evidence is needed for something to be considered 'expected' and when is it a 'concern'? The definition is liable to many different interpretations and poses a major challenge to address by industry and risk assessors.

Conclusion and recommendation (4)

The term 'relevant metabolite' is defined for chemical pesticides. Although used successfully in the evaluation of chemicals, this term is not applicable to microbial metabolites. It ignores the biological/ecological properties and roles of secondary metabolites. Based on the argument above, we question whether the term 'relevant metabolite' should at all be used in the data requirements for MCAs.

The term 'relevant metabolite' seems firmly embedded in the regulation texts. Once revision of the data requirements is initiated, it would be timely to remove the term or to replace it with one which is more lucid in its meaning.

4.2. Confusing requests for secondary metabolites in many data requirements

Several requirements for micro-organisms raise questions on

secondary metabolites (Table 3). Often, the focus of the individual data requirements is primarily on properties of the MCA, with references to secondary metabolites almost being incidental. Only Data requirement 2.8 specifically requests information on the production of secondary metabolites. Overall, this has resulted in the duplication of information being requested. The terminology of the texts varies between individual data requirements, resulting in confusion. The non-uniform use of the term "metabolite" (Table 3, third column) is compounding the problem.

Data requirement 2.8 focuses on *known* toxins with unacceptable effects explicitly stating that information should only be provided when known toxins are formed by related strains of the same species (Table 4). The rationale is that an MCA may theoretically produce harmful toxins known to be produced by phylogenetically related species. The other data requirements do not make reference to known metabolites, but many focus on toxins. This explains why regulatory experts request information on metabolites in general and not just the known metabolites as requested in Data requirement 2.8 (Tables 3 and 4).

Conclusion and recommendation (5)

Only Data requirement 2.8 is specifically requesting 'information on the production of metabolites (especially toxins)' and on 'known' toxins. In several other data requirements, which focus on the MCA properties, information on any toxic secondary metabolite is requested in addition. In these data requirements different terminology is used which is liable to different interpretations. The current confusion could be mitigated by rewriting the data requirements, using well defined and unambiguous terminology.

5. Secondary metabolites under different regulations

Much attention is given to secondary metabolites of MCAs but the EU regulatory system overlooks the fact that the same species occur naturally as part of the soil or plant “microbiome”. The microbiome consists of complex, dynamic populations of disparate epi- and endophytic micro-organisms. They produce a plethora of known and unknown bioactive metabolites, yet this has never been perceived as posing any kind of risk as opposed to metabolites produced by MCAs.

Comparison with other types of organisms which also generate secondary metabolites is of interest in this respect. For example, the plant-based biopesticides, the “botanicals”, are useful plant protection products that depend on an array of active metabolites. However, in the current guidelines for botanicals, these metabolites are treated differently compared to MCA metabolites (EU, 2014; FAO/WHO, 2017; OECD, 2017). It is notable that not once do these guidance documents, in those parts treating the botanicals, mention the word “metabolite”. Definitions relating to “metabolite” production have instead been replaced with the concept “component of concern”, defined as “any component which has an inherent capacity to cause an adverse effect on humans, animals or the environment and is present or is produced in a plant protection product in sufficient concentration to present risk of such an effect.” This definition disregards the type of metabolic pathways that leads to production of a compound. From a risk perspective, this seems a sound approach, since the essential issue is the potential for adverse (including toxic) effects. It is of subordinate importance if a compound (of concern) is a primary metabolite, a secondary metabolite, a polymer or something else.

Conclusion and recommendation (6)

Production of secondary metabolites is not specific to micro-organisms, as plants and other groups of organisms collectively produce a multitude of secondary metabolites. Clearly, micro-organisms and their metabolites (primary or secondary) are integral parts of the natural biota to which humans are continuously exposed.

When put into this context, current risk assessment of secondary metabolites of MCAs appears to be disproportionate and needs to be reconsidered.

6. Current and proposed risk assessment approach

6.1. Current secondary metabolite toxicity endpoint and exposure data issues

A wide range of toxicity tests and endpoints are available for determining toxicity of microbial metabolites to non-target organisms. However, the designs and endpoints of most tests have been generated for other purposes than pest control. Scientists designing these tests include natural product chemists prospecting for new lead compounds to develop into therapeutics, pathologists seeking to elucidate the role of secondary metabolites in pathogenesis/antagonism and toxicologists wishing to determine the potential impact of such compounds on humans. Since much of this data is available in the public domain, applicants can base their assessment on these findings, even though the tests and/or endpoints may not be pertinent to MCAs.

Examples of potentially irrelevant endpoints are the *in vitro* inhibition of mammalian cancer cell growth by metabolites of *Beauveria bassiana* (Mudgal et al., 2013), cytotoxicity observed in *in vitro* tests with boar semen (Hackl et al., 2015) or increase in the incidence of micronucleated erythrocytes at high dose only (EFSA, 2017). Such tests indicate that certain (high) amounts of a secondary metabolite could be cytotoxic *in vitro*, however, these results cannot be easily extrapolated to intact animals and relevant environmental exposures. It can be

questioned whether such non-OECD, non-data requirement tests should trigger animal testing.

6.2. Proposed risk assessment approach

Future data requirements and guidance on secondary metabolites should differentiate better between toxigenic and beneficial micro-organisms and aim to categorise these according to the risks they pose. This will require a good understanding of the evolution and ecological role of these groups of micro-organisms. Such guidance will allow for more informed risk assessments to be made and accelerate the whole process. Progress in differentiating between these groups of micro-organisms is presented in a recent OECD review on microbial secondary metabolites (OECD, 2018a).

The USA practices a hazard-based approach: an MCA is considered to have little or no risk if tests and literature reviews show no indication that it produces toxins in quantities that could be deleterious when the MCA is deployed as a plant protection product (OECD, 2018b). The product can then be admitted to the market.

Two EU funded projects, RAFBCA and REBECA (Strasser and Pernfuss, 2005; Strasser et al., 2011, 2008) suggested inclusion of some tests purely to provide that kind of reassurance that the secondary metabolites pose little risk (e.g. (Garrido-Jurado et al., 2016; Skrobek and Butt, 2005)). However, except for the AMES (genotox) and ecotox (e.g. *Daphna*, *Artemia*) tests, the other tests proposed (e.g. tests against animal cell lines) still have to be confirmed as acceptable by the regulatory authorities for risk assessment of MCAs. In selected tests the potential toxin production by an MCA can be evaluated by using crude extracts from culture filtrates that would contain the widest range of metabolites the micro-organism could ever produce *in vitro* (RAFBCA and REBECA recommendation). This approach has several highly commendable attributes. It is largely indifferent to variations in culture conditions and isolates and would take into account any additive or synergistic interactions between different compounds. Furthermore, it represents the “worst case scenario” since the metabolites are more concentrated than they would be in natural systems. If tests have a clear negative result crude extracts can be a useful tool. However, if there are (geno)toxic effects seen with the crude extract the problem arises that a) it is unknown which toxin is producing the effect, and b) it is very difficult to translate the (high) doses in those lab tests to the actual *in situ* field exposure. So in cases of effects, the crude extracts may unnecessarily complicate the risk assessment.

Conclusion and recommendation (7)

There is a need for clear toxicity and exposure data requirements based on agreed tests and endpoints reflecting the actual (*in situ*-field) risk. We conclude that a more hazard-based approach (practiced in other parts of the world) for evaluation of potential production of toxins by an MCA, can be a good way forward. The production of unknown, potentially toxic, substances does not need to be further considered, if a basic hazard assessment is passed without any concerns.

The aim should be to do as few toxicology tests as possible. For known toxins, the focus should be on the substance itself. For unknowns, and in perspective of updated data requirements, tests are not recommended. A thorough literature search might be sufficient to conclude that unknown toxins are not an issue. Based on the outcomes, a revised strategy for tests/investigations can be established. The crude extract approach, for example, could be suitable in situations where it is considered motivated to “screen” for unknowns.

7. What is the way forward?

In this review we address several topics related to the EU data

requirements of secondary metabolites and pinpoint specific issues that cause difficulties in the risk assessment. The sources of the problems are diverse and have a strong historical component. Current data requirements and evaluations of secondary metabolites from MCAs are based on chemical principles and have a poor foundation in microbiological science. We show in this paper that the current EU data requirements relating to secondary metabolites are inconsistent and more complicated than necessary to apply in risk assessment. Unfortunately, many of the difficulties persist almost 20 years after their preparation. Looking at the problems retrospectively gave us clues to their simplification, which is urgently needed.

In short, our primary recommendations are:

- New data requirements for microbial plant protection products must be based on the biological and ecological properties and hazards of micro-organisms.
- Measuring secondary metabolites *in situ* is not useful, unless a known toxin is produced.
- There is clearly a need to redraft the data requirements for secondary metabolites of MCAs to make them more pertinent to micro-organisms, using well-defined and unambiguous terminology. Lucid, robust definitions are required which can distinguish between metabolites and breakdown products from either microbial or chemical sources. There is also a need to increase the awareness among regulatory experts, risk assessors and applicants of the differences between metabolites from microbial and synthetic origin.
- The term and concept 'relevant metabolite', used successfully in the evaluation of chemical pesticides, is not applicable to microbial metabolites and causes much confusion. We propose that this term should not be used in data requirements for MCAs.
- We propose that revised data requirements for MCAs adopt a more hazard-based approach for evaluation of the potential toxin production of MCAs. Given that a basic hazard assessment is passed without any concerns, the production of unknown, potentially toxic, substances does not need to be further investigated.

CRedit authorship contribution statement

Jacqueline W.A. Scheepmaker: Conceptualization, Investigation, Writing - review & editing, Visualization, Project administration, Supervision. **Marloes Busschers:** Conceptualization, Investigation, Writing - review & editing. **Ingvar Sundh:** Conceptualization, Investigation, Writing - review & editing. **Jørgen Eilenberg:** Investigation, Writing - review & editing. **Tariq M. Butt:** Conceptualization, Investigation, Writing - review & editing, Visualization, Funding acquisition.

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