Microbiome-aware ecotoxicology: relevance, pitfalls and challenges

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Abstract

The diversity and significance of host-associated microbial communities has emerged over the last 15 years to the point when some may now assume that "Nothing in Biology makes sense except in the light of the microbiome". The advent of high-throughput 'omics' techniques has revealed the multiple roles and interactions occurring among hosts, their microbial partners and their environment, and has radically changed our views of biology, evolution and individuality. Sit at the interface between a host and its environment, the microbiome is a dynamic interface that responds to both host and environmental factors, for better or worse. For this reason, the microbiome now appears as a relevant, yet understudied compartment for ecotoxicology research. Various examples show that the microbiome reacts to and interacts with contaminants, with consequences for hosts and the ecosystem, and great advances can be expected from studies in which microbiome research meets toxicology. Yet, for the encounter to be scientifically productive, caution is needed in study design, inferences and extrapolations. In this paper, we emphasize the relevance and challenges of microbiome research in environmental toxicology through documented examples, and draw readers attention to pitfalls that should not be overlooked when producing and analyzing their data. We advocate for the development of a "microbiome-aware ecotoxicology".

1. Introduction: the microbiome is relevant to environmental toxicology

The significance of microbes to larger organisms is long documented, yet it is the advent of high-throughput sequencing technologies which ultimately revealed how diverse and numerically abundant they were. Microorganisms form complex symbiotic communities of eukaryotes, bacteria, archaea, and viruses referred to as the microbiota or microbiome (HMP 2012; McFall-Ngai et al. 2013). Over the last 15 years, the microbiome has been a new frontier in Life Sciences (Alivisatos et al. 2015). Microbiome community compositions can now be assessed through sequencing of marker genes for identification (e.g. variable regions of the ribosomal RNA-encoding genes) or metagenomes to address functions (genes present in the community), and compared to allow hypothesis testing (Ramette 2007). Microorganisms are involved in many functions including nutrition, defense, immunity, development and behavior (Archie and Theis 2011; McFall-Ngai et al. 2013). The microbiome is not only beneficial, but necessary for the proper development and functioning of multi-cellular organisms (Rakoff-Nahoum et al. 2004). In humans, the microbiome

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may represent as many cells as the hosts and up to 1,000 times more genes, questioning the concept of individuality and the limits of self, leading researchers to view a host as an ecosystem colonized by microorganisms (Gilbert et al. 2012; Rees et al. 2018). The holobiont concept, referring to the entity formed by a host organism and its various microbial associates, arouse to encompass the complexity of hosts and their microbiome (Mindell 1992; Bordenstein and Theis 2015). All these discoveries have fueled a "microbiome revolution" that increasingly spreads through all fields of Life Sciences, with extensions to behavioral and Human sciences (Blaser 2014; Cryan and Dinan 2019).

Recent research focuses mostly on the links between hosts and their microbiome, and the reciprocal influence they exert on each other, revealing its significance to host physiology, homeostasis and disease/health (Selber-Hnatiw et al. 2017). Interestingly, most members of the microbiome are located on epithelia (mucosa, skin...), *i.e.* animal or plant polarized tissues that separate the inside from the outside of the organism. Sit at the interface between a host and its environment, the microbiome can thus be influenced by the latter, and may be the hosts buffer and first line of defense against environmental stressors (Barr et al. 2013). This is the case in deep-sea hydrothermal vent mussels, which harbor bacteria able to oxidize hydrogen sulfide in their gills, their abundances varying depending on exposure levels (Halary et al. 2008). To date, toxicology studies which aim at investigating the effects of chemical compounds on organisms examine the accumulation, bio-transformation and elimination and effects in tissues, and rarely account for the microbiome despite its self-evident relevance. This paper aims to emphasize the relevance, pitfalls and promises of microbiome research for ecotoxicology and advocates for the emergence of a "microbiome-aware ecotoxicology", *i.e.* an approach that fully incorporates the microbiome compartment as a dynamic interface interacting with host and environment (Figure 1).

2. The microbiome responds to and interacts with contaminants

Data on effects of environmental contaminants on microbiota has been published over the last decade, with a focus on animal gut-associated bacteria (Claus et al. 2016; Jin et al. 2017; Rosenfeld 2017; Adamovsky et al. 2018). Studies usually consist in controlled exposure performed on model organisms. Duration is chosen appropriate to the organism's developmental cycle, and contaminants concentrations are usually ecologically relevant. In most studies, the composition of bacterial communities is assessed using 16S rRNA metabarcoding. Many studies include complementary analyses on host parameters of toxicological relevance such as markers of the immune system, tissue histology, developmental markers (abnormalities…). Less studies do investigate activities of microbiome members on contaminants, and bidirectional interactions (Figure 2; Schmidt et al. 2019).

The body of evidence gathered includes exposure to various types of contaminant including pesticides, antibiotics, heavy metals, xenobiotics, or nanoparticles (Licht and Bahl 2019; Monroy-Torres et al. 2019). Most published results indicate that gut bacterial community composition is modified during these exposures, supporting that environmental chemicals interfere with microbiome composition (Evariste et al. 2019). Species richness (estimated by number of Operational Taxonomic Units or OTUs) usually drops, and the relative abundances of some bacterial taxa vary. Another major finding, best supported by data on vertebrate models, is that the gut microbiota interacts with contaminants (Figure 2). Various human gut bacteria for example metabolize native contaminants or those conjugated in the liver, and thus prevent the effects of some deleterious compounds (Claus et al. 2016). Alternatively, the microbiome may activate compounds and mediate toxicity to hosts. Human colon microbiota was for example shown to convert polycyclic aromatic hydrocarbons into estrogenic metabolites (Van de Wiele et al. 2005); and the nephrotoxicity of melamine in rats is mediated by the bacterium *Klebsiella terrigena* that generates cyanuric acid from melamine (Zheng

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et al. 2013). Bacterial activation is also reported in pharmaceutical applications (Hasegawa 2004). Contaminants also interfere with activities of the gut microbiota (Licht and Bahl 2019). Because of these multiple interactions, the microbiome must now be considered a key player in toxicology.

3. Producing and interpreting microbiome data relevant to ecotoxicology

3.1. Conceptual pitfalls: identifying a "good" microbiome and a "good" model species

Ecotoxicology studies that address the microbiome rely on a microbial ecology background, and thus need to consider the caveats associated with this discipline. Experimental investigations to date have focused mostly on bacteria. However, various studies have demonstrated the significance of Archaea and microbial Eukaryotes including fungi to hosts physiology (Hoffmann et al. 2013; Richard and Sokol 2019), and the key role of phages in regulating bacterial populations (Mirzaei and Maurice 2017; Bettarel et al. 2018). A comprehensive description of the microbiome functioning will emerge only when all components are accounted for (Rowan-Nash et al. 2019). However, the lack of universal, easy-to-obtain markers for some groups including viruses, still precludes the development of systematic analyses, that require deep metagenomics and specific expert analysis pipelines.

Another point to keep in mind is that OTUs composition provides only a partial description of the real microbial diversity. A diverse bacterial population could indeed correspond to a single 16S rRNA-based OTU, yet could include clearly distinct genotypes, potentially quite divergent in term of their respective functional phenotypes and different in their responses to a stimulus (Andam 2019; Hanafiah and Lopes 2020). The genus *Vibrio* for example encompasses strains with very different lifestyles including commensals, light-producing mutualists of the squid *Euprymna scolopes*, and pathogens of numerous metazoans, despite displaying almost identical 16S rRNA sequences (Sawabe et al. 2007). Lack of variation in an OTU relative abundance level could thus hide a rebalancing of its genotypic and phenotypic composition (see 4.1).

A major difficulty is the general lack of baseline knowledge regarding microbiomes of toxicology model species, for which very little-to-no data is available regarding wild populations (Uenishi et al. 2007; Hird 2017; Shinohara et al. 2019). Besides, organisms used in tests are often sourced from rearing facilities. They have thus experienced domestication, a process documented to lead to massive changes in bacterial microbiome compositions in vertebrates, including species richness decrease and shift in taxa abundances (Alessandri et al. 2019). In humans, comparative analyses of populations showing distinct levels of "modernization" in their lifestyle for example indicate that the gut microbiota diversity decreases with increased "modernization" (Blaser 2016; Sonnenburg and Sonnenburg 2019). Effects in other taxa such as arthropods are less documented and less clear-cut (e. g. Chen et al. 2018)). Whatsoever, contrary to most genetically-encoded physiological traits, microbiomes change rapidly upon domestication, and the representativity of a model versus its wild relatives and its relevance should always be questioned in the light of its domestication history. Lack of knowledge, along with inter-individual variability (discussed below, 3.2), undermines the identification of the 'normal', balanced microbiome composition, i.e. the eubiotic state. This compromises the proper diagnosis of a dysbiosis (an 'abnormal', unbalanced) state upon exposure to contaminants (Figure 2). Indeed, although many factors can cause dysbiosis that may lead to health issues, it is not so easy to establish what a healthy/eubiotic microbiome is (Iebba et al. 2016).

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In between the relative simplicity of most invertebrate-associated microbiota in which a few OTUs are usually dominant (e.g. Raymann et al. 2017) and the extreme complexity of mammal-associated microbiota (with hundreds to thousands of OTUs), teleost fish and their tens to a few hundred bacterial OTUs (Llewellyn et al. 2014) offer an interesting intermediate, besides their relevance to the monitoring of aquatic ecosystems. Choosing a model thus involves addressing different levels of microbiome complexity. Because of these domestication- and complexity-related issues, whether current models in toxicology are any relevant to microbiome-aware ecotoxicology studies needs to be evaluated.

3.2. Technical pitfalls: performing the right experiment to detect effects

As for any microbiology study, uncontrolled exposure to microorganisms will influence the outcome of microbiome studies. This is why most studies use controlled microcosms and monitoring of various compartments (*e.g.* food for animals, water for aquatic organisms). When scaling up to more holistic approach such as mesocosms or the natural environment, the number of required controls dramatically increases.

Fifteen years of human gut microbiome research revealed the high level of intra- (between body regions or life stages) and inter-individual heterogeneity in community compositions (HMP 2012; Rothschild et al. 2018). Although less documented in other taxa, high levels of intra- and inter-individual variation are for example found in teleost fish species of economic value including Atlantic cod, salmon and Rainbow trout, and in zebrafish and medaka maintained in lab conditions (Star *et al.* 2013; Llewellyn *et al.* 2014; Lowrey *et al.* 2015; Duperron *et al.* 2019; Evariste *et al.* 2019). This level of variability makes the identification of treatment-induced trends harder to detect, requiring replication levels that considerably increase the cost of study. It also requires ascertaining that the exact same tissue region is investigated in all specimens. Overall, a trade-off has to be decided between microbiome complexity, number of tested conditions, replication level and sequencing depth required to demonstrate an eventual effect.

3.3. Correlation versus causality: confounding factors should not be overlooked

Many studies assume that modification in microbiome community structure directly results from the action of contaminants. This is evident in experiments involving antibiotics (Dethlefsen and Relman 2011; Blaser 2016) but less so in most cases in which effects can be indirect. For instance, dietary emulsifiers disrupt the multi-layered mucus structure which harbors the microbiome and participates the epithelial barrier in the human gut. The resulting mucosa degradation allows bacterial encroachment, indirectly leading to a shift in microbiome composition, and triggering inflammatory-associated diseases (Chassaing et al. 2015). Host compounds produced during inflammation may also modify the microbiome (Ormsby et al. 2019). Bacterial products can also destabilize the whole community as illustrated in the case of Crohn's disease where propionic acid promotes the growth of a virulent phenotype of *Escherichia coli* (Ormsby et al. 2020).

Overlooked factors may also influence the outcome of experiments, including various life history traits. Sex is documented to influence both gut microbiota composition (Bolnick et al. 2014; Haro et al. 2016), as well as the microbiome responses. Exposure to silver nanoparticles was for example shown to modify the gut microbiota structure of male but not female zebrafish (Ma et al. 2018). Interestingly, sex-differentiated responses to compounds are also commonly reported in classical toxicology studies, for example in medaka fish exposed to cyanotoxins (Le Manach et al. 2016).

4. The roads less traveled: challenges in microbiome-aware ecotoxicology

Besides responses of community compositions to contaminants, the field needs to move on and link community structure to functions, to become quantitative, and to investigate temporal patterns (Figure 2). For this, it can build on tools and approaches developed in other domains of microbiome research.

4.1. Functionality and integration

One major finding of the Human Microbiome Project was that the bacterial communities taxonomic compositions were highly variable among body regions and among individuals, but that the functions they performed, as encoded by the metagenome, were highly conserved among individuals and tissues (HMP, 2012). Similar functions were thus performed by taxonomically distinct microorganisms. This concept known as functional redundancy today is recognized as key to the resistance and resilience of microbial communities (Allison and Martiny 2008; Moya and Ferrer 2016). As previously mentioned (3.1), closely related bacteria on the other hand can display markedly different functionalities. Community composition alone is thus a poor predictor of functions, and identity and functions should be investigated in tandem. Functional capabilities can be evaluated through metagenomic sequencing, but genes (and functions) that are actually expressed are better evaluated by metatranscriptomic or metaproteomic approaches. Metabolomics, which map metabolites, are another important tool that profiles the ongoing metabolism (Bundy et al. 2009; Gao et al. 2017) although, as for all of the above, the improvement of databases will be critical (Gertsman and Barshop 2018). The integration of these approaches in multi-omics appears challenging, yet particularly promising for evaluating how the microbiome may mitigate or amplify toxic effects (Rohart et al. 2017).

4.2. Quantification

Going quantitative is a challenging task. On the microbiome side, communities may shift rapidly and non-linearly between contrasting alternative, more or less stable states provided a set of parameters reaches threshold values. The existence of yet-undescribed tipping points is hypothesized to explain the existence of bimodal distributions of abundances of certain bacteria in the human gut (Lahti et al. 2014; van Nes et al. 2016), and may be responsible for the onset of dysbiosis. An aim of microbiome-aware ecotoxicology should be to identify contaminant threshold values relevant to microbiomes, in a way similar to what is done for toxicological effect on hosts traits, for example the determination of non-observable adverse effect limit (NOAEL). For this, studies should examine dose-dependent responses and chronic exposure to low doses, as done in toxicology studies. This requires preliminary documentation of the plasticity of the microbiome compositions under controlled conditions.

Microbiome composition assessments also need to become quantitative. Metabarcoding datasets produce taxa relative abundances tables, and their variations, while absolute abundances are rarely informed. An increase in one group thus cannot be properly interpreted, as it may as well represent a lower decrease relative to other groups in a globally shrinking population. Assessment of the biological significance of changes thus needs to rely on absolute, not relative abundances. Antibiotics for example reportedly affect relative abundances of taxa, but most of all they affect the total number of bacteria present, which is probably the main way antibiotics influence microbiome functioning (Vieira-Silva et al. 2019). Quantifying bacteria associated with organisms is challenging, as demonstrated by the very different estimations of bacteria-to-human cell ratios found in the

literature (Sender et al. 2016), but tools are available, including quantitative/real time PCR, that may at least roughly inform densities (Tkacz et al. 2018).

4.3. Temporality and resilience

The nature and amplitude of variations are important aspects of microbiome response to contaminants. Composition of communities and diversity indices are still the main endpoints of most studies. However, the dynamics of these variations during and after exposure are certainly as important. In humans, these dynamics are individual-dependent (Flores et al. 2014). Dynamics inform resilience, evaluating whether variations have long-term effects on the microbiome, or whether it fully recovers and returns to a naive, pre-exposure stable state (Figure 2). Antibiotic exposure was shown to affect human gut bacterial communities for several months post-exposure, and similar effects can be expected with other contaminants (Dethlefsen and Relman 2011; Francino 2015). Whether iterative exposure to contaminants may lead to habituation, and thus become less influential to microbiomes, also remains to be properly tested.

4.4. Interactions and prediction

The holobiont is more than just the sum of its parts (Bordenstein and Theis 2015). With dozens-to-thousands distinct coexisting bacterial taxa, and many more if eukaryotes, archaea and phages are considered, an animal's gut or skin is a whole ecosystem in which multiple interactions among members and with the host influence its functioning. Interactions with the environment, including the contaminants and microorganisms occurring there, also need to be accounted for. Co-occurrence networks that are based on positive or negative correlations between the occurrence of partners, functions, and environmental features help in exploring these interactions and formulate hypotheses (reviewed in (Faust and Raes 2012). A strong relationship between the presence of a contaminant and that of certain bacterial taxa for example suggests an ability to metabolize the former, which can then be tested (Claus, Guillou, and Ellero-Simatos 2016). Changes in the network structure itself can indicate microbial successions in time series experiments, or dysbiosis (Zhou et al. 2010). Networks may inform modelling approaches as illustrated in a study that modeled the dynamics of marine phages (Hoffmann et al. 2007).

5. Conclusion: what can the microbiome do for ecotoxicology and vice versa?

By analogy with the famous essay by Dobzhansky (1973), it is tempting these days to suggest that "Nothing in Biology makes sense except in the light of the microbiome". Ecotoxicology is no exception to this trend, and must not lag behind other disciplines that have embraced the microbiome revolution. However, the microbiome is not just another ecotoxicological endpoint, but a peculiar and complex biological compartment that exhibits its own ecological, metabolic, functional and thus ecotoxicological rules (Evariste et al. 2019). Instead, a microbiome-aware ecotoxicology needs to develop (Figure 1). This involves questioning, and not only transferring, classical toxicology protocols and model organisms' relevance to microbiome studies. Close cooperation between microbial ecologists and ecotoxicologists is needed. They have a lot in common: the complexity of microbiomes and their response mirrors that of contaminants and their interactions; and both domains start with reductionist approaches, and strive to scale up to holistic approaches that encompass systems full complexity and produce real-life-relevant data.

A major challenge is to move on from observing correlations to addressing causality, and ultimately explain processes, e.g. demonstrate mitigating effects of the microbiome. Repeatability is

a key point, which involves inter-studies comparisons and meta-analyses for which tools are becoming available (e.g. Amplicon Sequence Variants for OTU clustering (Callahan et al. 2017)). With this in mind, microbiome features including taxa or functions may become bioindicators of contamination, as recently proposed in stream ecosystems (Simonin et al. 2019). Modelling interactions between environment, contaminants, microbiomes and hosts will become tractable, with a certain level of predictive power (Gould et al. 2018). No doubt the dialogue between disciplines will result in mutual enrichment, and will allow to make the most of the microbiome revolution applied to ecotoxicology.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

All authors (SD, SH, AG and BH) have contributed to the writing of the manuscript and the production of figures. All authors have read and approved the final version.

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Figures

Figure 1: Ecotoxicology studies the effect of chemicals on organisms at the population level. A microbiome-aware ecotoxicology perspective acknowledges the importance of microorganisms associated to larger organisms in their hosts biology, at the level of individuals as well populations. It integrates the microbiome as an element of the system, and adapts protocols to investigate toxicological effects at each level.

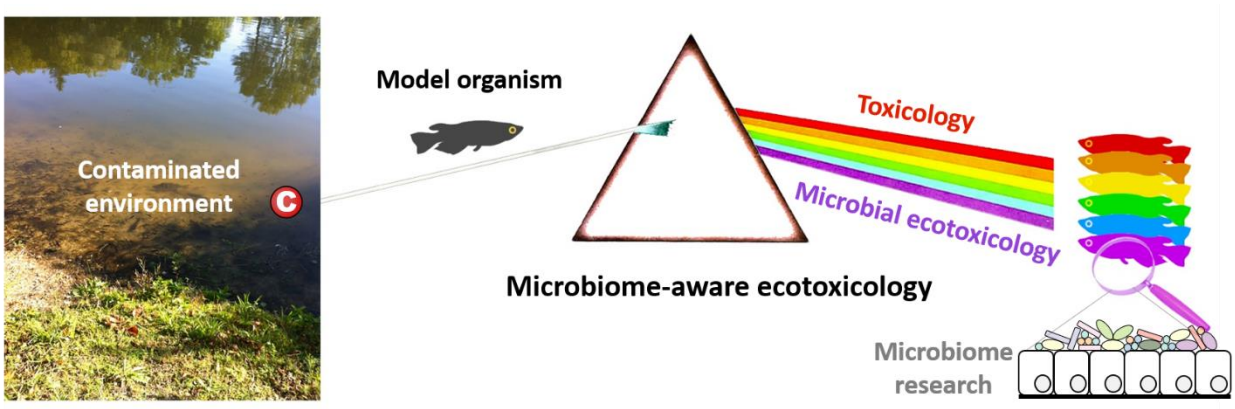

Figure 2: Sit at the interface between the environment and the host, the microbiome may interact with contaminants. Sequestration, inactivation and degradation mitigate potential effects on host health, while activation or potentialisation on the other hand reinforce the effect of contaminants. The microbiome composition, abundance and functions themselves respond to exposure. Dysbiosis can occur. Post-exposure recovery ultimately leads to a new stable state, identical or altered compared to the pre-exposure state. Promising future lines of research are emphasized (see text section 4).