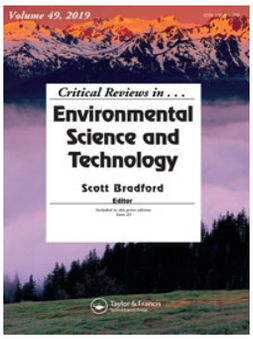

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Implications of microbial adaptation for the assessment of environmental persistence of chemicals

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ABSTRACT

Persistency of organic chemicals is a key property in their environmental risk assessment. Information on persistency is often derived from the results of biodegradability screening tests such as the ready biodegradability tests (RBTs). RBTs are, however, not designed for this purpose and suffer from several problems that lead to a high variability of the results and, hence, to difficulties in their interpretation. The origin and exposure history of the inocula used for biodegradability testing can lead to highly variable outcomes. Microbial adaptation to chemicals and its impact on biodegradation needs further investigation in order to have a better understanding of their effects on persistency assessments of chemicals. It is well described that microbial adaptation stimulates biodegradation of organic chemicals. Several mechanisms responsible for these phenomena have been described, amongst which are i) shifts in community composition or abundances, ii) mutations within populations, iii) horizontal gene transfer or iv) recombination events. These adaptation processes may well be mimicked under laboratory conditions, but the outcome remains difficult to predict as we lack a fundamental understanding of the adaptive responses. This review aims to bring together our current knowledge regarding microbial adaptation and its implication for the testing of biodegradation of chemicals.

KEYWORDS

Adaptation; biodegradability testing; biodegradation; microbial community; organic pollutants; persistence

Abbreviations

BHR	Broad host range plasmid
HGT	Horizontal gene transfer
L-GLDA	L-Glutamate-N,N-diacetate
NTA	nitrilotriacetic acid
OECD	Organization for Economic Co-operation and Development
RBTs	Ready biodegradability tests

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QACs Quaternary ammonium compounds
WWTP Waste water treatment plant

1. Introduction

Microbial degradation is one of the major processes that affects persistence of chemical pollutants in the environment (Alexander, 1981). The terms biotransformation or biodegradation refer to enzyme-catalyzed reactions that result in the structural modification of an organic chemical (primary biodegradation) or its complete breakdown to CO_2 and water (ultimate biodegradation) (Kolvenbach, Helbling, Kohler, & Corvini, 2014). Microbial biodegradation of organic chemicals may occur either via growth-linked degradation or via co-metabolism. Growth-linked biodegradation, also called metabolic degradation, is the process where microorganism use the chemical as sole carbon and free energy source (Liu, Helbling, Kohler, & Smets, 2014; Tran, Urase, Ngo, Hu, & Ong, 2013). Co-metabolic degradation, on the other hand, is the process where the chemical is metabolized in cells that grow on another type of carbon and free energy source, usually a compound with biochemical features that resemble the chemical (Kassotaki, Buttiglieri, Ferrando-Climent, Rodriguez-Roda, & Pijuan, 2016; Peng, Qu, Luo, & Jia, 2014; Tran et al., 2013).

The biodegradation rate of a new chemical is one of the key parameters used for estimating its environmental risk, along with physico-chemical degradation and partitioning processes (Boethling et al., 2009). Under the European REACH regulation (Registration, Evaluation, Authorization of Chemical substances), biodegradability and persistency is determined by a range of laboratory-based methods, developed by the Organization for Economic Co-operation and Development (OECD) (OECD, 1992; Thouand, Durand, Maul, Gancet, & Blok, 2011). Most of them are set up for growth-linked biodegradation in an aquatic medium, often using bacteria sampled in wastewater treatment plants or surface water, and do not take potential co-metabolic degradation events into account (Kowalczyk et al., 2015; van Ginkel et al., 2008).

Ready biodegradability tests (RBTs) differ from higher tier simulation tests as they have been designed as simple and inexpensive methods to identify readily biodegradable chemicals. These tests are so stringent that it is assumed that a compound giving a positive result, according to the RBTs guideline, will rapidly and completely be biodegraded in aquatic environments under aerobic conditions (Comber & Holt, 2010). Negative or positive outcomes cannot, however, be taken as proof that the tested compound is persistent or not in the real environment (Painter, 2002), where conditions and exposure history may be very different from the laboratory screening tests. A readily biodegradable chemical, according to

RBTs, can be persistent in the environment, if for example, this chemical appears to not be bioavailable for the local microbial communities or if the pH, oxygen concentration or temperature conditions are not suitable for active biodegradation (Kowalczyk et al., 2015). Moreover, the parameters measured by the test itself can be a source of confusion between ready biodegradability and non-persistency. RBTs are conducted under aerobic conditions with relatively high concentrations of the test substance, in the range of 2 to 100 mg/l, as sole carbon and free energy source. A defined buffered medium, that contains various salts and ammonium as nitrogen source, is used as incubation medium. RBTs rely on measurement of general metabolic activities like O_2 consumption or CO_2 production to quantify mineralization of the chemical (OECD, 1992, 2006). As such, the parameters measured in these tests are not a straightforward measurement of the degradation of the chemical as they do not provide information about the biotransformation pathway of the molecule itself. Hence, the test results can be, and often are, misinterpreted. (Trautwein & Kümmerer, 2011), since a chemical that is transformed in the test but not mineralized could be considered as non readily biodegradable, if CO_2 production was the measured parameter, but may have a short half-life in the environment. Domestic sewage, activated sludge or secondary effluents are typically used as source of microorganisms (inoculum) for the biodegradation testing. RBTs do not reflect environmental conditions and are not realistic, as they cannot predict mineralization rates under environmentally relevant conditions (Federle, Gasior, & Nuck, 1997), and can only identify easily biodegradable chemicals in batch culture. Furthermore, extensive research has demonstrated that RBTs (including the OECD series) suffer from several problems that lead to a high variability in the testing results and to difficulties in their interpretation (Dick, Rey, Boschung, Miffon, & Seyfried, 2016; Howard & Banerjee, 1984; Kowalczyk et al., 2015; Thouand et al., 2011; van Ginkel et al., 2008). Some of these variabilities are understood and can be attributed to differences in specific factors, such as the temperature or the oxygen concentration (Greskowiak, Hamann, Burke, & Massmann, 2017). However, other factors affecting the biodegradation rate in RBTs, such as the physico-chemical conditions and the quantity and quality of the inoculum, remain currently unresolved and still need to be addressed (Greskowiak et al., 2017; Kowalczyk et al., 2015). It is evident that these factors should be identified and further investigated in order to minimize any potential effects of them on the test outcomes.

One of the major reported factors causing variability in the RBTs is the source of the inoculum (Dick et al., 2016; Martin et al., 2017; Mezzanotte, Bertani, Innocenti, & Tosin, 2005; Thouand, Capdeville, & Block, 1996; Thouand et al., 2011; Vázquez-Rodríguez, Garabétian, & Rols, 2007). The

quality of the inoculum is affected by i) the total cell density (Blok & Booy, 1984; Martin et al., 2017; Thouand et al., 1995; Vázquez-Rodríguez et al., 2007), ii) the diversity of the community (Forney et al., 2001), iii) the ratio between nutrients and biomass (F/M ratio) (Vazquez-Rodriguez, Palluy, Goma, & Rols, 1999), and iv) the origin and exposure history (Itrich et al., 2015; Kim et al., 2017; Mezzanotte et al., 2005; Thouand et al., 1996).

The origin and history of an inoculum determines its composition and diversity as the result of a long evolutionary process of successive microbial adaptations (Itrich et al., 2015; Kowalczyk et al., 2015). Microorganisms are able to develop mechanisms of resistance and metabolism once exposed to novel environmental pollutants (van der Meer, 2006). Well known examples are the evolution of resistance mechanisms to metals or antibiotics in the natural environment of microbial species (Bergeron, Boopathy, Nathaniel, Corbin, & LaFleur, 2015; Bouki, Venieri, & Diamadopoulos, 2013; Di Cesare, Fontaneto, Doppelbauer, & Corno, 2016; Huerta et al., 2013; Lo Giudice, Casella, Bruni, & Michaud, 2013; Martin, Bass, & Liss, 2015; Petrie, Barden, & Kasprzyk-Hordern, 2015). Bacteria are subject to evolution in response to environmental perturbations, which may lead to the development of novel enzymes and even novel catabolic pathways (Kolvenbach et al., 2014; van der Meer, 2006). It may be hypothesized that the occurrence of cells that metabolize rare chemicals is the result of community adaptation to exposure to this chemical or a structurally related compound (van der Meer, 2006). As a result, members of the community can increasingly withstand the potential toxic effects of chemicals or even degrade them (Lo Giudice et al., 2013; Saez, Aparicio, Amoroso, & Benimeli, 2015; Zhuang, Tay, Yi, & Tay, 2005). Therefore, microbial adaptation and microbial evolution can be crucially important when assessing the biodegradation of chemicals (Comber & Holt, 2010; Itrich et al., 2015; Markiewicz et al., 2011). More importantly, it explains why the variability in different biodegradability tests yields a low predictive value for the environmental persistency of chemicals (Thouand et al., 2011). It also becomes evident why results of RBTs change over time as microbial populations apparently adapt within years or even months to metabolize chemicals which were previously considered to be persistent (Itrich et al., 2015). Finaly, it could also explain the hight percentage of chemicals, not yet released in the environment, that tend to fail the test, as the inoculum has not been exposed to the chemical yet (Painter, 2002). Several studies show an improvement of the biodegradation process even after a short period of pre-exposure to the tested chemical (Chong & Lin, 2007; Elcey & Kunhi, 2010; Ferro Orozco, Lobo, Contreras, & Zaritzky, 2013; Kim et al., 2017; Manonmani, Chandrashekaraiah, Sreedhar Reddy, Elcey, & Kunhi, 2000; Saez et al., 2015).

Here, we will review what is currently known about microbial adaptation and its implication for the biodegradation of chemicals in the environment. We will also explore how this knowledge can be used to improve the consistency of biodegradation testing. Investigating the relationship between microbial adaptation and biodegradation will ultimately facilitate the design of more robust screening tests. Furthermore, directed microbial adaptation may also be a solution to improve bioremediation of recalcitrant chemicals (Duan, He, Li, & Li, 2015; Timmis & Pieper, 1999) in contaminated environments or in wastewater treatment plant. Important issues and terms that we will discuss are 1) the definition of adaptation, 2) role of exposure to environmental pollution in microbial adaptation, 3) experimental induction of adaptation, 4) mechanisms of adaptation relevant to biodegradation, and 5) integrating microbial adaptation in biodegradability testing approaches.

2. Definition of adaptation

In respect to biodegradation, adaptation can be defined as an evolutionary process in which a change in the microbial community or organisms takes place and which is manifested by an increase in the biodegradation rate of a chemical (Itrich et al., 2015; Kowalczyk et al., 2015; Wiggins, Jones, & Alexander, 1987). Of course, in other fields microbial adaptation may be defined differently, such as in the field of eukaryotic host-microorganism interactions (Ochman & Moran, 2001; van der Meer & Sentchilo, 2003). However, this review will focus on the microbial adaptation leading to biodegradation. Adaptation is complex as it refers to phenomena that may take place both in individual cells and at the community level (Itrich et al., 2015; van der Meer, de Vos, Harayama, & Zehnder, 1992). As will be described later, the phenomena are interconnected and could be used to classify chemicals based on the adaptation level in RBTs. Sometimes, acclimation and acclimation period are also used in addition to adaptation and adaptation period if these modifications appear during the life time of the organism and/or after a short-term exposure (Chong & Lin, 2007; Karahan, Olmez-Hanci, Arslan-Alaton, & Orhon, 2010; Kim et al., 2017; Liao, Li, Zou, Xie, & Yuan, 2016; Saez et al., 2015). Nevertheless, acclimation also refers to the period required for the development of an optimum microbial community before they start vigorous biodegradation in laboratory tests (Howard & Banerjee, 1984; Jones, Martinez, Maroo, Deshpande, & Boswell, 2004; Wiggins et al., 1987). During this phase, the microbial community undergoes a series of enzyme induction processes and resulting biochemical changes in order to initiate the biodegradation of a specific substrate (Jones et al., 2004). In other words, acclimation is a term derived from laboratory observation after deliberate pre-exposure of the inoculum to a chemical or mixture of chemicals (Ferro Orozco et al., 2013; Jiang, Luo, Yan, & Tay,

2009; Manonmani et al., 2000), while adaptation is a term that refers to environmental observation of the same process after a long-term exposure, typically long enough to modify the community composition.

Microorganisms may display different degrees of plasticity with respect to changing their physiology and biochemistry at different levels suggesting that adaptation may well happen in many different environments and by many types of mechanism or combinations thereof (Aelion, Swindoll, & Pfaender, 1987; van der Meer, 2006). These will be explored later in this paper. At the community level, biodiversity and species abundances can be affected by chemicals (van der Meer, 2006). At the level of the individual cell, adaptation can refer either to phenotypic or genetic adaption.

3. Role of exposure to environmental pollution in microbial adaptation

Historically, several studies have shown the occurrence of microbial adaptation in the environment after short or long periods of exposure to a xenobiotic chemical (Itrich et al., 2015; van der Meer, 2006). This process is usually demonstrated experimentally by comparing biodegradation studies in contaminated and in pristine sites (Itrich et al., 2015).

A major condition leading to adaptation of bacteria and resulting biodegradation of a specific chemical is the presence of the chemical itself or a structural analog in the environment. Indeed, experimental studies have shown that pre-exposure of a community to a defined chemical increases the chance of having an efficient degradation of this molecule by adaptation (Alidina, Li, & Drewes, 2014; Oh, Tandukar, Pavlostathis, Chain, & Konstantinidis, 2013; Pfaender, Shimp, & Larson, 1985; Thouand et al., 1996). Moreover, if such a chemical serves as a stable food supply, development of a degrader population for that niche will therefore lead to an adaptation at a community level (van der Meer, 2006). Toxicity of chemicals has also been shown to stimulate microbial adaptation (Lima-Morales et al., 2016; Oh et al., 2013).

Microbial adaptation has been observed in a variety of contaminated environments, such as aquifers (Aelion et al., 1987; Aelion, Dobbins, & Pfaender, 1989; Alidina et al., 2014), fresh water (Schwab, Maruscik, Palmisano, & Ventullo, 1992), (Larson & Davidson, 1982), estuaries (Pfaender et al., 1985), wastewater treatment plants plants (Chonova et al., 2016; Itrich et al., 2015), sediment (Shimp, 1989) and soil (Macedo, Neu, Kuhlicke, & Abraham, 2006). Below, we present five examples of directed microbial adaptation that are well described in the literature.

a. Quaternary ammonium compounds (QACs), which are cationic surfactants, induce bacterial adaptation leading to increased capacities to biodegrade QACs. It became evident that pre-exposure in various

ecosystems, such as in periphytic communities in fresh water (Schwab et al., 1992), aquatic sediment (Shimp, 1989), fresh water (Oh et al., 2013) and soil (Tezel & Pavlostathis, 2015), increased resistance and adaptation of bacterial community members to QACs as a result of either selection of degrading species (Oh et al., 2013) or by recombination events, such as horizontal transfer of plasmids or integrons (Tezel & Pavlostathis, 2015).

b. Adaptation to and increased biodegradation of nitrilotriacetic acid (NTA), has been reported by comparison of NTA degrading capacities in samples from a pristine river and a river exposed to NTA for several years (Larson & Davidson, 1982). Another investigation has shown that estuarine bacteria can also adapt to the presence of NTA in their environment and degrade the compound after relatively short lag periods (Pfaender et al., 1985).

c. Decades ago, the herbicide atrazine was sometimes included as reference substance in persistency testing as it was considered truly persistent (Ingerslev & Nyholm, 2000). A more recent study, however, has shown that atrazine is biodegraded in some RBTs (Lapertot & Pulgarin, 2006). In yet another study (Satsuma, 2009) an atrazine degrading-community has even be isolated from a river ecosystem (Comber & Holt, 2010). Adaptation of the microbial populations to this compound has resulted in a shift towards atrazine biodegradation rate in an aquatic environment (de Souza, Seffernick, Martinez, Sadowsky, & Wackett, 1998; Yale, Sapp, Sinclair, & Moir, 2017). However, it has also been reported that atrazine is still detected in high concentrations in soil, even after 22 years after the start of the observations (Jablonowski, Köppchen, Hofmann, Schäffer, & Burauel, 2009). This work highlights the fact that atrazine is particularly persistent in soil and that microbial adaptation to this compound might not occur in every ecosystem or under all conditions. This may well be the result of differences in bioavailability of the compound, of differences in specific growth conditions, or of differences in metabolic potential within the bacterial community.

d. Adaptation of soil communities to some congeners of polychlorinated biphenyls (PCBs) has also been observed (Macedo et al., 2006). Growth of a microbial biofilm on a highly contaminated soil leads to an increase in the transformation rate of PCBs, in comparison to a biofilm community without history of exposure. Furthermore, biofilms derived from a highly polluted site were the only ones able to transform pentachlorinated congeners (Macedo et al., 2006). In addition, genes involved in chlorobiphenyl degradation were exchanged between introduced bacteria and indigenous biphenyl degraders (Focht, Searles, & Koh, 1996).

Degrading genes encoding proteins such as the biphenyl dioxygenases can be promoted by the presence of PCBs and PAH in the ecosystem, leading to local adaptation of the exposed microbial community (Hoostal & Bouzat, 2016). Genetic exchange of genes encoding PCB and chlorobiphenyl degrading proteins are well known, and are the results of plasmid transfer between species (Top & Springael, 2003).

e. L-Glutamate-N,N-diacetate (L-GLDA) is a recent example of adaptation of the inoculum to a specific chemical in wastewater treatment plant (Itrich et al., 2015). L-GLDA, a chemical introduced in 2010 in the U.S.A, has been used as a model to study the spread of microbial adaptation across a large geographic region. Previous work conducted in the Netherlands (van Ginkel, Geerts, & Nguyen, 2005), has shown that this molecule can be considered as readily biodegradable. This was concluded from degradation studies using inocula from a wastewater treatment plant and surface water from the Rhine river according to the OECD 301 guidelines. Moreover, a bacterial strain using L-GLDA as the sole nitrogen, carbon and energy source has been isolated from Dutch activated sludge by the same researchers. However, OECD 301B RBTs using inocula from several wastewater treatment plants in the Midwest and the Mid-Atlantic (U.S.A.) prior to the introduction of L-GLDA on the American consumer market showed contradictory results. L-GLDA was considered not readily biodegradable, with a degradation level below 12% after 28 days of incubation. This divergence in biodegradation results between American and Dutch inocula supports the view about the influence of inoculum properties on biodegradation testing results. Moreover, these results have contributed to the investigation and demonstration of potential adaptation processes occurring in activated sludge, both in laboratory experiments and in actual wastewater treatment plants. After introduction of L-GLDA in the U.S.A. L-GLDA was readily biodegradable using inocula from 12 different wastewater treatment plants (WWTP) after 22 months of extensive use. This investigation documented a field adaptation to a new chemical and demonstrated the importance of implementing adaptation of bacterial communities in persistency testing strategies.

Environmental adaptation is a complex process where the outcome is hard to predict but should nevertheless be considered if persistent chemicals are to be identified reliably. The greatest uncertainty concerns, among other factors, estimations of the time required for adaptation to a new molecule on the one hand and the parameters that promote the adaptation process on the other hand (van der Meer, 2006). Others environmental factors, such as the chemical bioavailability, the type of available energy

sources and the toxicity of the substance are also known sources of uncertainty concerning environmental persistency.

4. Experimental induction of adaptation

Induction of microbial adaptation by long and short-term exposure to chemicals is a crucial step to study underlying mechanisms of adaptation under laboratory conditions. As microorganisms are perfect models for evolution experiments, many investigations have been conducted to study their evolution dynamics (Elena & Lenski, 2003). One approach to measure both the temporal pattern and magnitude of microbial adaptation is to use time dependent perturbation experiments. In this approach, biodegradation capacity and fitness of adapted communities and their cells are compared with the original inoculum (Koskella & Vos, 2015). Many of these laboratory studies revealed adaptation to and biodegradation of a specific chemical (Stephenson, Lester, & Perry, 1984). Moreover, adaptation under laboratory conditions i) reduces the risk of error in biodegradability testing (Thouand et al., 1996), ii) limits the potential toxic effects on bacteria and microalgae (Wang et al., 2016) and iii) enhances the biodegradation of environmental contaminants (Wang et al., 2016).

Table 1 gives an overview of laboratory investigations of microbial adaptation which led to an improvement of biodegradation. These findings show that biodegradation of many chemicals has been investigated using different kinds of inoculum and treatments (Table 1). The dominant ones are pre-exposure to the tested compound or to related compounds and induction of adaptation by mutation.

Several investigations have been conducted to point to the role of pre-exposure leading to adaptation of the inoculum in bioremediation processes. Adaptation times in these experiments ranged from days (Saez et al., 2015) to weeks (Toräng & Nyholm, 2005), depending on the type of inoculum, molecules being assessed and of the experimental conditions. Pre-exposure experiments can be conducted in batch system, semi-continuous systems (e.g., fed-batch) or continuous culture systems. Each of these systems has their own advantages and disadvantages. Batch culture systems are easy to set up, reproducible, controllable and they allow analyses of the fate of a specific molecule in time. Semi-continuous systems are more complex and can be used for long term culturing to allow the enrichment of species involved in biodegradation in an enrichment purpose, in order to select one specific population or microorganism in the cultivated community. Continuous culture systems, such as chemostats, are complex and expensive systems suitable to study environmental adaptation processes in the laboratory under controlled growth conditions that, to a certain extent,

Table 1. Overview of investigations on microbial adaptation in laboratory systems.

Tested Chemical	Inoculum	Inoculum origin	Treatment	Results	References
Phenol	Anaerobic sludge community	WWTP (Lund, Sweden)	Pre-exposure to phenol	Community shift linked to enhanced biodegradation	(Guieysse, Wikström, Forsman, & Mattiasson, 2001)
	Pseudochrobactrum sp XF1	Activated sludge from coking WWTP (Xuzhou, China)	Induced mutation by UV irradiation	Complete degradation of phenol	(Mao et al., 2015)
	Mixed aquatic microbial community	Lake water (Lake Michie, Durham, D.C, U.S.A)	Exposure to phenol in continuous flow microcosms	Enhanced biodegradation of phenol and structurally related compounds	(Shimp & Pfaender, 1987)
	Activated sludge community	Activated sludge	Exposure in membrane bioreactor (MBR)	Enhanced biodegradation	(Boonnorat, Chiemchaisri, Chiemchaisri, & Yamamoto, 2014)
p-Nitrophenol	*Pseudomonas putida*	Activated sludge from WWTP (Maxeville, France)	Pre-exposure in sequential batch culture	Increase in the concentration of degraders. Decrease variation between results	(Thouand et al., 1996)
2.4.5-Trichlorophenol	Activated sludge community	Activated sludge from WWTP (U.S.A)	Pre-exposure in batch culture	Adaptation to biodegradation at 10 and 20 μM	(Marsolek, Kirisits, & Rittmann, 2007)
Antraquinone (Dye AB48)	*Pseudomonas stutzeri*. CECT 930	Spanish type culture collection (ATCC 17588)	Exposed to a ionic liquid 1-ethyl-3-methyl imidazolium ethylsulfate	Enhanced biodegradation of the tested molecule (Dye AB48)	(Alvarez, Deive, Angeles Sanroman, & Rodriguez, 2016)
Azo dye	*Bacillus* sp.	Isolated from industry effluent (Pallavaram, Chenai, India)	Pre-Exposure in batch	Biodegradation enhancement	(Gopinath, Asan, Muthukumar, & Velan, 2009)
			Induced mutation		(Gopinath, Murugesan, Abraham, & Muthukumar, 2009)
	Pseudomonas stutzeri. CECT 930	Spanish type culture collection (ATCC 17588)	Exposed to a ionic liquid 1-ethyl-3-methyl imidazolium ethylsulfate	Enhanced biodegradation of the tested molecule (Dye RB5)	(Alvarez et al., 2016)
Soak liquor	Environmental halotolerant bacterial strain	Environmental samples (India)	Induced mutation by nitrous acid & UV	Biodegradation enhancement with mutation induced by nitrous acid. No significant difference for UV induction.	(Sekar et al., 2009)

(*continued*)

Table 1. Continued.

Tested Chemical	Inoculum	Inoculum origin	Treatment	Results	References
Hexachlorocyclohexane (X-HCH)	Soil community	Sugar cane field (India)	Long term enrichment to R-HCH	Biodegradation enhancement	(Elcey & Kunhi, 2010)
	Soil and sewage community	India		Complete mineralization	(Manonmani et al., 2000)
Lindane	Streptomyces consortium	Isolated from sediment samples (Argentina)	Pre-exposure in sequential batch culture	Biodegradation enhancement. Decrease of toxicity.	(Saez et al., 2015)
	Sediment community				(Pesce & Wunderlin, 2004)
Quaternary ammonium compounds (QAC)	Sediment community	River sediment (USA)	Pre-exposure in fed-batch reactor	Degradation & mineralization of QAC and change in the microbial community	(Oh et al., 2013)
	River periphytic communities		Pre-exposed in model streams		(Schwab et al., 1992)
Aniline 4-Nitrophenol 4-Chloroaniline	Freshwater community	River surface water (Copenhagen, Denmark)	Semi-continuous preexposure procesure (SCEP)	Significant reduction of lag phase. Increase of reproducibility	(Toräng & Nyholm, 2005)
Levofloxacin	*Chlorela vulgaris*	Unknown (South Korea)	Pre-exposure in batch	Biodegradation enhancement	(Xiong, Kurade, & Jeon, 2017)
5-Tolytriazole	Activated sludge communities	Activated sludge from WWTP (Germany)	Semi-continuous batch culture	Reduction of lag phase	(Herzog, Yuan, Lemmer, Horn, & Müller, 2014)
Benzoate	Activated sludge communities	Activated sludge from WWTP (U.S.A)	Exposure in semi-continuous batch culture	Biodegradation enhancement	(Dennison, O'Brien, Gopalkrishnan, & Stark, 2010)
L-Glutamate-N,N-diacetate (L-GLDA)	Activated sludge communities	Activated sludge from various WWTP (U.S.A)	OECD 303A activated sludge WWTP simulation tests	Biodegradation enhancement	(Itrich et al., 2015)
4-Nitrotoluene	*Acidovorax* sp.	Unknown	Pre-exposure in successive batch culture	Ability to grown on the tested molecule via new degradation pathway	(Ju & Parales, 2011)
Tert-butyl alcohol	Activated sludge communities	Activated sludge from WWTP (Singapore)	Sequencing batch reactor (SBR)	Biodegradation enhancement	(Zhuang et al., 2005)
Bisphenol-A	Activated sludge communities	Aerobic laboratory-scale activated sludge reactor	Pre-exposure in consecutive batch assay	No significant change in BPA degradation between acclimated and non-acclimated	(Ferro Orozco et al., 2013)

mimic natural *in situ* conditions better than other culturing conditions (Gresham & Hong, 2015). Depending on the study and on the type of chemical, pre-exposure experiments can be conducted under environmentally realistic conditions by using concentrations of the tested chemical that are environmentally relevant and by mimicking the environmental conditions as best as possible.

Introduction of mutations is another example of treatment which can be undertaken at the laboratory level that leads to a microbial adaptation at a genetic level and to a biodegradation enhancement (Table 1). It has been shown that ultraviolet (UV) irradiation induces mutations in the DNA resulting in increased degradation efficiency of phenols (Mao, Yu, & Xin, 2015). Genetic mutations by UV irradiation seem to change genes encoding specific degrading enzymes resulting in enhanced activities of them. These irradiations can induce mutations by forming pyrimidine dimerization and cross-links in DNA, but mutations can also be induced by exposure to various physical (Duan et al., 2015) or chemical agents (Sekar, Sivaprakasam, & Mahadevan, 2009). However, even if an induced mutation leads to enhanced degradation of various pollutants, such as phenol (Sekar et al., 2009), azo dye (Joshi, Inamdar, Jadhav, & Govindwar, 2013), tannery soak liquor (Sekar et al., 2009) and oil (Chen, Yang, Huang, Zhang, & Ding, 2011; Duan et al., 2015), the randomness of the approach makes it difficult to use it in bioremediation applications or in biodegradation testing strategies. Moreover, these types of mutation and genotype evolution induced by external stress are complex and difficult to predict in natural environments.

As demonstrated by the data in Table 1, microbial adaptation towards the degradation of chemicals can be induced in the laboratory through many approaches. Regarding RBTs, adaptation induction techniques could play an important role in the development of more robust biodegradation tests through either the development of simulation tests or the production of standardized adapted inocula. More robust tests will be of benefit for environmental risk assessment as well as for industry and regulators. This will limit the number of studies yielding contradictory results and prioritize the use of higher tier tests for the chemicals of concern, leading to a better and more directed action and regulation. It should be noted that every study listed in Table 1 used different terms to describe the same processes or techniques, as for example acclimation and adaptation, which in these studies refer to the same process. However, among all techniques used to trigger adaptation events, pre-exposure of the inoculum seems to be one of the most studied ones and the best candidate to optimize the biodegradability test. However, it should be noted that most compounds presented in Table 1 are already considered as readily biodegradable or inherently

biodegradation by the standard RBTs, and that adaptation didn't change their persistency classification except for L-GLDA (Itrich et al., 2015). Moreover, most reported studies in Table 1 have investigated the biodegradation kinetics before and after adaptation without using standard tests. RBTs such as the OECD 301 series do not yield kinetic data as these can be influenced by many factors, such as biomass growth. The results reported in Table 1 cannot be used to draw conclusions about the potential effect of adaptation on persistent compounds, and whether pre-exposure will completely change the conclusion of the test. However, these data demonstrate that adaptation can be induced under certain laboratory conditions, even with persistent or inherently biodegradable compounds.

Before implementing adaptation strategies to biodegradability tests, there is a need to predict the effects of those strategies on composition and function of the microbial communities. For this purpose, we need to understand the diverse mechanisms of microbial adaptation and their implications for the biodegradation of organic chemicals. The next section will be focused on the diverse levels of microbial adaptation that need to be investigated before implementing pre-exposure in modified RBTs.

5. Mechanisms of adaptation relevant to biodegradation

Microorganism can adapt by many mechanisms or combinations thereof (Aelion et al., 1987; van der Meer, 2006). As previously mentioned, adaptation in the presence of persistent chemicals may occur at the community or single species level (van der Meer, 2006). Notably, these levels are interconnected, and cannot be regarded as separate entities or disentangled from each other (Koskella & Vos, 2015). To include induced adaptation in the biodegradability testing, we need to understand at what biological levels adaptation occurs. This will also help to discriminate chemicals based on the adaptation process induced by their presence and the expected frequency of these adaptations in the environment. As we will describe in this section, microbial community adaptation is controlled by three mechanisms; i) microbial interactions within the community and governed by microbial ecology concepts, ii) the genetic information that controls the functional potential of the whole community, and iii) the interplay between the microbial community and the environment. The challenge to improve our understanding ofmicrobial adaptation and how this can be included into biodegradation testing lies in the measure of key parameters relevant for the different levels of adaptation. Next, we will discuss microbial community adaptation and how community composition can influence persistency testing results.

5.1. Adaptation at the community level

Communities are defined as a group of multi-species organisms potentially interacting with each other and coexisting in space and time (Konopka, 2009; Nemergut, Shade, & Violle, 2014). Activated sludge communities are commonly used as inocula for RBTs although their composition and activity vary substantially [19] (Mezzanotte et al., 2005). Indeed, the presence or absence of specific species known to be efficient degraders should be measured to prevent any false results in RBTs. These tests are using the general metabolic activity, like O_2 consumption or CO_2 production, to quantify mineralization of the test chemical. If a small group of specific microbial species can use the tested molecules as sole source of carbon and free energy, and then produce CO_2 or O_2 in the process, the result of the test depends largely on the original community composition (Forney et al., 2001) and on its adaptation capacity. One proposed solution to prevent false results due to the absence or presence of specific species is to increase the cell density of the inocula used in RBTs (Thouand et al., 2011). Increasing cell density seems to be a good way to decrease the risk of variability in results (Martin et al., 2017; Thouand et al., 1995). However, this approach does not consider possible community changes over time and space and is based on the probability of the presence or absence of specific microbial species. Indeed, communities may vary both spatially and temporally (Nemergut et al., 2014). Seasonal variation in WWTP community composition (F. Ju, Guo, Ye, Xia, & Zhang, 2014) may shift test results because of the temporal and spatial scales over which microorganisms function. Investigating changes over time in community composition and function may lead to a more relevant and realistic prediction of chemical persistency in the environment (Kowalczyk et al., 2015). Moreover, the success of biodegradation is not only determined by the presence and activity of specific degraders, but also by the growth and dynamics of organisms with important secondary functions (Head, Jones, & Röling, 2006).

In order to study adaptation at a community level a combination of several techniques is needed, as most environmental species are as yet uncultivable (Koskella & Vos, 2015). As such, a mix of cultivation dependent approaches and multi-omics analyses may well be a good option to predict and follow the activity and succession of the used inoculum (Gutleben et al., 2018). Cultivation-independent methods to monitor the microbial community dynamics (e.g., taxonomic or functional diversity) coupled to modeling techniques can lead to the discovery of common patterns associated with the biodegradation of a molecule (Head et al., 2006). Recent studies have followed and characterized microbial communities after long-term exposure, using next-generation sequencing and targeting a set of catabolic genes involved in a particular biodegradation process (Lima-Morales et al.,

2016). These types of analyses are part of the growing field of community systems biology (Zengler & Palsson, 2012). Computational and mathematical modeling that use genome information to predict community behavior from a metabolic perspective is a challenging but also a promising approach for the comprehension of intrinsic community interactions and interplay with their environment (Gottstein, Olivier, Bruggeman, & Teusink, 2016; Hanemaaijer et al., 2015). Multi-omics analyses coupled to modeling approaches may lead to a better prediction of chemical persistency in the environment including information about community composition and behavior in the presence of the tested chemical. Most research to follow the adaptation of a community performed so far relied on conventional microbial ecology techniques, such as DGGE (Boonnorat et al., 2014) and 16s rRNA amplification sequencing (Zhuang et al., 2005). Other more complex analyses have been used to study microbial adaptation or to understand the differences of biodegradation between strains. For example, Grady et al (Grady et al., 2017) used a multi-omics approach to compare gene expression profiles of two *Pseudomonas aeruginosa* strains growing on n-alkanes. They used results from RNA-seq, microarray, ribosome footprinting and proteomics to compare gene expression profiles of the two strains and to explain why their growth rates differed significantly despite the fact that they had a high genome sequence identity. Divergence was explained by a difference in transcriptome and in the set of activated genes (Grady et al., 2017). Moreover, one of the strains that could consume n-alkanes more rapidly was able to up-regulate a specific small part of its genome, including operons responsible for alkaline proteases. The use of modern high-throughput multi-omic technologies allowed a simultaneously analyses of the entire cell system at the molecular level and thus reduces the probability of incorrect conclusions/assumptions being made. Among these multi-omics technologies, metatranscriptomics is another technique that can be used, with the adapted bioinformatics tool, to obtain the global transcriptome of an adapted microbial community. Modern metatranscriptomic techniques have proven to be very efficient to quantify the activity level of a specific degradation genes in a complex community, and to correlate this transcriptome with a transformation activity of the whole community (Helbling, Ackermann, Fenner, Kohler, & Johnson, 2012).

Changes of microbial community dynamics after exposure to chemicals may well be the result of a community adaptation to a new source of nutrients (Zhang et al., 2008). Thus, if no variation is observed in a microbial community after a perturbation, we should consider it as non-adapted (Nogales, Lanfranconi, Piña-Villalonga, & Bosch, 2011). Variations inside the community composition can alter the functionality of the community, that can lead to a change in the ecosystem services provided by microbial

communities (e.g. biodegradation capacity and participation in biogeochemical cycles) (Liao et al., 2016). Growth of specific subpopulations able to metabolize the substrate may change the community in its totality (van der Meer et al., 1992). However, community level adaptation is not only based on these processes, but also depends on microbe-microbe interactions. Adaptation of microbial communities in terms of composition and function can also co-occur jointly to an adaptation at the genetic level. We referred earlier in this paper to a study in which resistance and degradation of QACs has been investigated after long-term exposure in different bioreactors [55]. Results have shown that QACs exposure led to a decrease of the community taxonomic diversity and to the enrichment of a strain, *Pseudomonas nitroreducens*, which showed mutations in QACs catabolic genes and HGT events ultimately leading to its genetic adaptation (Oh et al., 2013). This specific study, amongst many others, provides evidence that microorganisms can adapt simultaneously both at a community and at a genetic level (Ayala-del-Río, Callister, Criddle, & Tiedje, 2004; Di Cesare et al., 2016; Lima-Morales et al., 2016; Tandukar, Oh, Tezel, Konstantinidis, & Pavlostathis, 2013; Top & Springael, 2003).

5.2. Adaptation at the genetic level

A second level of microbial adaptation is adaptation at the genetic level. Genetic adaptation has a significant impact on the biodegradation of chemicals and this phenomenon has been reported frequently (Das et al., 2015; Focht et al., 1996; Nojiri, Tsuda, Fukuda, & Kamagata, 2014; Springael & Top, 2004; van der Meer, 1994). Investigating microbial adaptation at the genetic level is a way to assess and follow the biodegradation capacity of an organism or a community in different environments. Information on the potential of an inoculum to degrade a certain chemical can be obtained by tracing the functional genes responsible for its degradation (Fang, Cai, Yu, & Zhang, 2013; Röling & van Verseveld, 2002). Presence or absence of specific catabolic genes, capacity of microorganism to perform horizontal gene transfer or environmental conditions promoting genetic mutations could be used to predict environmental persistency of chemicals in specific environments (Top, Springael, & Boon, 2002).

Genetic adaptation of microorganisms may be due to i) mutations (Hersh, Ponder, Hastings, & Rosenberg, 2004; Mao et al., 2015), ii) horizontal gene transfer (HGT) (Springael & Top, 2004; Top & Springael, 2003), iii) recombination events (Bhadbhade, Dhakephalkar, Sarnaik, & Kanekar, 2002; Chaguza, Cornick, & Everett, 2015), iv) gene duplications (Beacham, 1987) or v) combinations thereof (Nojiri et al., 2014). These modifications can affect the expression of gene function and could lead to

the acquisition or loss of function (Hottes et al., 2013; van der Meer, 2006) within the whole community.

Not all genetic modifications will ultimately result in microbial adaptation, as the majority of mutations are deleterious or neutral, but some of them may lead to tolerance of microorganisms (Bergeron et al., 2015; Bouki et al., 2013; Sharma & Thakur, 2009) and even to the biodegradation of certain chemicals (van der Meer, 2006). Genetic modification may play a prominent role in microbial diversification, and may affect microbial community assembly through selection (Nemergut et al., 2014). Analysis of the catabolic pathways of xenobiotic-degrading bacteria indicated that they might have adapted to the new chemicals by expressing new functions to resist their potential toxic effects or to use them as alternative source of essential nutrients, such as those involved in carbon, nitrogen or free energy metabolism (Top & Springael, 2003).

Mutations are a fundamental source of natural variation that drives evolutionary processes and they play a prominent role in bacterial adaptation to environmental pollutants (Ferenci, 2007; Gerrish, 2001; Hersh et al., 2004; van der Meer, 2006). Toxicity and genotoxicity of physical or chemical agents can promote the adaptation of microbial strains or other members of a community. As previously described, several researchers have studied mutation induction as a strategy to enhance the biodegradation of organic compounds. Indeed, alteration of gene sequences by exposure to various physical or chemical agents, such as UV irradiation and nitrous acid, can lead to the enrichment of effective strains for biodegradation of chemicals. Phenolic compounds (Ju & Parales, 2011; Mao et al., 2015), oil (Chen et al., 2011; Duan et al., 2015), azo dyes (Kannappan Panchamoorthy Gopinath et al., 2009; Joshi et al., 2013) or polycyclic aromatic hydrocarbons (Sideri, Goyal, Di Nardo, Tsotsou, & Gilardi, 2013) are different mixtures or chemicals that have been degraded by different strains after different stress induced mutations. Due to their randomness, such mutations can also lead to the biodegradation of nontoxic and persistent chemicals, even in an environment that provides enough nutrients for the community (van der Meer, 2006). Even if beneficial mutations are rare and can be lost by successive replication and random drift (Elena & Lenski, 2003), they can accumulate into the genetic material of the population over time. New catabolic pathways can be produced and production of specific enzymes can be increased by the accumulation of mutations (Lin, Yang, Xu, & Wu, 2011; Madigan et al., 2014). The implication of mutations into the dynamics of evolutionary adaption has been well documented (Elena & Lenski, 2003).

Metagenomic analyses of activated sludges, used as inoculum in RBTs, has shown a high prevalence of biodegradation genes for a number of

organic compounds (Fang et al., 2013). These genes are widespread and can be easily transferred across the whole community by HGT (also referred to as lateral gene transfer). HGT plays an important role in fitness and adaptation of bacterial communities in contaminated environments (Kweon et al., 2015; Top & Springael, 2003). Under particular conditions, individual cells that were involved in HGT events may be positively selected and expand the basis of bacterial adaptation (Nielsen, Bøhn, & Townsend, 2014).

HGT of catabolic genes can lead to the acquisition of detoxification pathways or can allow strains to gain alternative carbon, nitrogen, or free energy sources (Top & Springael, 2003). These mechanisms may be favored by stress factors, such as environmental pollution (Villegas-Torres, Bedoya-Reina, Salazar, Vives-Florez, & Dussan, 2011). However, a vast majority of HGT events in communities will be lost due to lack of replication success of the transferred material (Nielsen et al., 2014). The importance of gene transfer for adaptation to new compounds has been investigated in many studies (Janssen, Dinkla, Poelarends, & Terpstra, 2005; van der Meer et al., 1992) and these events have been observed for the breakdown of many compounds, such as chlorobenzene (van der Meer, 2006), atrazine (Aislabie, Bej, Ryburn, Lloyd, & Wilkins, 2005; de Souza et al., 1998; Janssen et al., 2005; Rousseaux, Soulas, & Hartmann, 2002), hexachlorocyclohexanes (van der Meer, 2006), other halogenated molecules (Janssen et al., 2005) and polycyclic aromatic hydrocarbons (PAHs) (Kweon et al., 2015).

Genetic recombination is an important mechanism employed by bacteria to rapidly adapt to selective pressures such as environmental pollution (Cordero & Polz, 2014; Thavamani et al., 2017; Top & Springael, 2003). In many cases, transfer of genes involved in biodegradation occurred due to plasmid exchange through conjugation events (Janssen et al., 2005; Top et al., 2002; van der Meer & Sentchilo, 2003). Other mobile elements, such as conjugative transposons and genomic islands also seem to play an important role (Top & Springael, 2003; van der Meer & Sentchilo, 2003).

Conjugation events allow intra- and interspecies transfer of plasmids with genes encoding antibiotic resistance or novel biodegradation pathways (van der Meer et al., 1992). Among all plasmid groups, the IncP plasmid-group seems to be involved in a wide range of catabolic and resistant functions (Tezel & Pavlostathis, 2015). The IncP plasmid group is one of the best characterized and described so called broad host range (BHR) plasmids, which are known to be responsible for the transfer of resistance and degradation genes in a wide range of hosts (De Gelder, Williams, Ponciano, Sota, & Top, 2008; Martin et al., 2015). The genes involved in the degradation of organophosphorus insecticides (Bhadbhade et al., 2002;

Horne, Sutherland, Harcourt, Russell, & Oakeshott, 2002), carbaryl (Hashimoto et al., 2006; Singh, Trivedi, & Phale, 2013), toluene and naphthalene (Kishida, Inoue, Ohtsubo, Nagata, & Tsuda, 2016), chlorophenols (Ma, Quan, Yang, & Li, 2012; Trefault et al., 2004), 2,4,6-trichlorophenol (Ma et al., 2012) and atrazine (Aislabie et al., 2005; de Souza et al., 1998; Rousseaux et al., 2002) are also contained on BHR plasmids. Plasmid-encoded pathways are advantageous for microbial communities because they provide genetically flexible systems and can be maintained in the population and transferred between bacterial species by conjugation (Sayler, Hooper, Layton, & King, 1990). However, isolation and characterization of BHR plasmids from the environments needs further investigation in order to fully understand their importance in the adaptation process (Li et al., 2015).

As mentioned before, microbial adaptation to a new chemical is not only driven by a shift in the community composition or in the genetic material. Microbial interactions and environmental conditions may also change the way the genetic potential is expressed and therefore change the phenotype of individual cells on the short term and that of the community on the long term.

5.3. Phenotypic adaptation

Phenotypic adaptation is the result of a modification in the expression profiles of genes involved in the degradation pathway of an organic chemical (van der Meer, 2006). Response at a transcriptomic level to an environmental stress can also be considered as biochemical adaptation or phenotypic plasticity (Koskella & Vos, 2015; van der Meer, 2006). Changes in gene expression are important processes in adaptation to abiotic and biotic environmental changes, and studies demonstrate that they are involved in adaptive evolution as well (Schulte, 2004). Phenotypic variation has been shown to allow a fraction of a genetically homogeneous population to survive after exposure to an antimicrobial compound (Balaban, 2004). This difference in response between the different sub-populations correlated to a global phenotypic heterogeneity of the populations. Furthermore, a certain phenotype of a *Rhodococcus opacus* strain has shown a higher capacity to degrade several chlorinated phenols compounds after dormancy (Solyanikova, Mulyukin, Suzina, El-Registan, & Golovleva, 2011). In this study, phenotypes were isolated using mineral medium with a single chemical as the sole carbon and free energy source. However, in the literature, phenotypic adaptation usually refers to changes in gene expression (van der Meer, 2006) and is not always considered as an adaptation mechanism. This phenomenon could impact the RBT results in a way that the inocula

in the test are transferred from their native environment to a batch culture with different physico-chemical conditions, possibly leading to a modification in catabolic gene expression. For example, the incubation temperature has been shown to influence the biodegradation rate of several organic compounds, (Lotto, Calil, Guedes, & Rosa, 2004; Sui et al., 2016). Moreover, even if the test conditions are the same, two supposedly identical inocula may have different responses due to phenotypic heterogeneity between them (Ackermann, 2015). In a recent review phenotypic heterogeneity is described as functional divergence between identical individuals living in the same environment [152]. We can assume that if divergence in phenotype can occur at an individual level, the same type of functional divergence due to phenotypic adaptation can occur at higher levels of biological organization. Microbial communities with a phenotype pre-adapted to the test condition may be promoted in comparison to a community that is non-adapted. Rather than the global biodegradation capacity of a community, the phenotypic adaptation could also influence the lag-phase of growth and activity of the community. This reflects the time required for the development of an optimum microbial community, in terms of biomass formation, enzyme production and pathway reconstrution, before they start vigorous biodegradation in laboratory studies (Jones et al., 2004; Wiggins et al., 1987). As such, phenotypic adaptation will mostly influence the biodegradation lag-phase of the tested chemical in RBTs. Due to its complexity, only a few papers have described the role of this process in the general adaptation phenomena of microbial communities and hence relevance to biodegradation tests.

In conclusion, before including strategies to promote adaptation in RBT we need to better understand and control the different levels of adaptation in order to predict its role in the environment and improve the relevance of the test.

6. Potential for including adaptation in biodegradability testing

Pre-exposure of the inoculum to the tested chemical prior to any test has been proposed as a method to enhance the biodegradability testing. However, using a pre-exposed inoculum is not allowed under the current ECHA guidelines, neither is it allowed to artificially increase the biomass density of the inoculum. Increasing the biomass to an environmentally relevant inoculum concentration has been shown to improve to reliability of RBTs (Martin et al., 2017). Furthermore, as mentioned earlier, pre-exposure can reduce the variability of degradability tests (Thouand et al., 1996) and could be an efficient method to include adaptation to RBTs. Another possibility is to prolong the test duration beyond 28 days and to increase the

volume of the test vessels, as recommended in the enhanced screening tests guideline (ECETOC, 2007). Enhanced biodegradation screening tests have been developed as complementary tests to the RBTs and more complex simulation tests. Running the test beyond 28 days allows for a longer acclimation period and hence more time for the inoculum to adapt to the test conditions. However, the use of adapted inocula from contaminated sites or inocula pre-exposed in the laboratory is not allowed during both RBTs and enhanced screening tests, because adapted inocula are currently considered as artificial microbial communities that do not reflect the natural environments. As discussed in this paper, most inocula are already exposed to many chemicals currently present in the environment. In fact, under the current state of global pollution, it can be argued that there are no pristine environments devoid of background contamination and consequently using unadapted microbial will not reflect the potential for biodegradation of the community. Pre-exposure and using adapted inocula may change degradation kinetics of the chemicals but could be considered within the persistency classification of chemicals (i.e. as probability of degradation occurring in the environment rather than classifications based solely on half-lives).

Including adaptation strategies in biodegradability testing will allow a better prediction of and may provide additional information about the persistency of compounds present at very low levels in the environment and for new chemicals. However, adaptation should be included without losing the stringency philosophy of the RBTs and should therefore be included in persistency assessments as an additional tier of testing. Inducing adaptation at environmentally realistic concentrations might seem more acceptable but would not be representative of the test conditions and may limit the extent and rate of adaptation. Several papers have reported that adaptation can be triggered only after that the concentration of a specific compound reaches a minimum threshold concentration (Spain & Van Veld, 1983; Toräng, Nyholm, & Albrechtsen, 2003). At lower concentrations, adaptation does not take place, not even after a long term exposure. If this is observed in a risk assessment context the threshold concentration would have to be related to the chemical release scenarios, bioaccumulation and toxicity profiles to assess if this raises significant environmental concerns.

It is, however, important to recognize that the objective of including adaptation is not to allow every persistent compound to pass RBTs, but to decrease the number of false negatives and the variability of test results. A key objective is to reduce the probability of chemicals being misidentified as non-biodegradable/recalcitrant in the environment. It has been proposed to classify the chemicals failing standard RBTs, but passing the test after pre-exposure, as “biodegradable with pre-exposed inocula” (Painter, 2002). Furthermore, a chemical degraded in RBTs with pre-exposed inoculum

should also be degraded in the activated sludge simulation test or in other simulation tests. It would also be an important consideration in the identification of potentially persistent mobile and toxic chemicals depending whether the release scenarios would lead to the potential for microbial adaption to occur. However, even if pre-exposure shows promising results in RBTs, validation and standardization of procedures are needed before including any deliberately exposed inoculum. Several concerns have been highlighted by the scientific and regulatory community which include i) ecological significance of adaptation, ii) the timescales required for adaptation to occur and iii) its relevance and importance for different habitats. It has therefore been proposed to include certain conditions, such as i) the original inoculum should be derived from a relevant habitat used for the persistence assessment, ii) environmentally realistic conditions are used for the pre-exposure conditions and a time frame (ECETOC, 2007).

In order to implement adaptation strategies to the biodegradability testing, we do not propose to modify the preexisting RBTs, but to extend the test and to use at least two different inocula. In this extended test, an inoculum from a relevant local environmental source (e.g., activated sludge, surface water, sediment, etc.) could be used after pre-exposure, to determine the (readily) biodegradability of the test compound by a naturally adapted inoculum. The time of adaptation needs to be determined. It has been reported that if the pre-exposure period, for wastewater treatment plan communities, is not more than 18 days, no chemical will pass a screening test with a pre-exposed inoculum without also being able to pass an activated sludge simulation test (Painter, 2002). Within these 18 days, catabolic pathway can be activated and phenotypic adaptation can occur, whilst after a longer period of time, there is a risk that adaptation reach both genomic and community levels. For practical purposes, exposure times used in the tests should last from days to a week. The exposure period could be similar to the pre-conditioning period, as described in the test protocol (OECD, 2006) (e.g. 5-7 days). Pre-exposure should be conducted under conditions similar to those in the test, and at either an environmentally realistic concentration or at the test concentration. Another realistic protocol would be to run two RBTs in series (Comber & Holt, 2010; ECETOC, 2007; Kowalczyk et al., 2015). The first test would be a standard one, without pre-exposure, while the second one would use the inoculum exposed for at least 28 days, which is longer than 18 days of pre-exposure. This solution would have the advantage of limiting the extra costs and risks of a pre-exposure test. The produced data would be compared with the regular RBTs that will remain unmodified and without pre-exposure. In the second part of the extended test, a highly adapted inoculum could be used to determine the persistence of chemicals failing the RBTs. This inoculum

would be standardized and highly adapted in the laboratory to the chemical family of the test compound, using a (semi)continuous culturing system. We believe that the differences in response between the different inocula will give valuable information about the environmental fate of the tested compound. If degradation is observed in both cases, then the molecule may be regarded as biodegradable. On the other hand, if the exposed inoculum shows degradation capacity and the environmental inoculum does not, we can conclude that the chemical is potentially (inherently) biodegradable after microbial adaptation (Comber & Holt, 2010). Such chemicals can be considered as non-readily biodegradable as well as non-persistent if a compound is only degraded by a highly adapted inoculum, it would confirm the capacity of microorganisms to consume this compound and the existence of a catabolic pathway, under certain condition. This chemical could therefore be considered as non-persistent, with possibility for bioremediation. Finally, a chemical that is non-degradable in every case can be classified as truly persistent (i.e. recalcitrant). However, if adaptation occurs and the tested chemical is only degraded after exposure, more advanced simulation tests should be conducted to conclude about the persistency in the environment. Moreover, not all inocula will adapt in the same way or at the same rate. Therefore, if contradictory results are observed, only the lowest degradation results should be considered for further analysis unless enough data were available to allow a statistical analysis of the frequency of degradation events.

Moreover, if possible, details should be given about the type and level of adaptation produced by the pre-exposure. Two chemicals leading to a different type of adaptation of the same inocula might be classified in different sub-categories. Investigating every possible level of adaptation would be time consuming and expensive, but it would provide valuable additional information on the environmental fate of the chemical. The kinetic and available –omic (Kowalczyk et al., 2015) information could be used to determine if the pre-exposure led to the expression of a preexisting pathway or to the development of a new one. Reduction of the lag-phase might be a consequence of induction of a preexisting metabolic pathway, while elimination of a chemical previously persistent might be the sign of the development of a new pathway, due to beneficial mutation, meaning that this adaptation process is also controlled by a random mutation factor. Furthermore, a community shift, measured by community profiling (e.g. DGGE or amplicon sequencing) would classify a chemical as a niche energy source, as only a sub-population of rare degraders present in the original inoculum were able to consume it. On the other hand, transcriptome analyses, such as RNA-Seq and DNA microarray, would provide information about the level of activity of specific catabolic genes. Transcriptomic

changes, after exposure, would classify a chemical as potentially degraded in the environment under favorable conditions. For example, this chemical might not be bioavailable or it could be present at too low concentrations and subject to competition with other chemicals. Finally, the last class would regroup all chemicals that induce adaptation at several levels or at the genomic level. As described in this review, every level of adaptation is interconnected, and it can be difficult to separate them. Furthermore, it would be difficult to discriminate chemicals inducing a genomic change, as these modifications can be hard to detect through metagenomics analysis, and have a random aspect. In a tiered testing approach, more tests could be conducted including the possibility to include adaptation in RBTs or in enhanced biodegradability tests. Recent innovations and approaches should also be considered to develop a more intelligent testing strategy in the context of persistency assessments. During the past years, studies have been initiated with the objective to improve the RBTs and biodegradability testing (Kowalczyk et al., 2015), using in-vitro and in silico approaches (Lombardo et al., 2014). For example, the research conducted by the CEFIC LRI ECO 11 project aimed at improving the OECD 306 screening test by increasing the test durations and investigating the impact of biomass density and diversity on the probability of biodegradability (Ott et al., 2019). Another CEFIC-LRI ECO 29 project that started in 2015 aimed at investigating the feasibility of using continuous culture systems to implement microbial adaptation to RBTs. This project uses a very specific experimental approach but other projects using modeling or other experimental approaches should be undertaken to develop and deliver more robust methods for assessing persistency of novel chemicals.

7. Conclusions

Improvement of the biodegradability assessment of organic chemicals is required to increase its relevance for environmental protection. Evaluating environmental biodegradability of a chemical is complicated, considering all the variables that can affect the final result. However, current RBTs need improvement to reliably assess biodegradability under environmentally realistic conditions and to avoid chemicals being falsely classified as persistent. They are designed to be stringent but simple and inexpensive tests, but they are not truly standardized and have a high potential to produce conflicting and variable results. Including approaches to enhance microbial adaptation potential in the persistency testing strategy is one of the solutions proposed by the scientific community to improve the value of RBTs. As we discuss in this paper, changes in the biodegradation rate of organic chemicals over time should not be discounted, given the prodigious

adaptation capacity of microbial communities to new chemicals. Microbial adaptation has been observed in almost every environment and at several biological levels and can be induced in the laboratory with relative ease. However, this phenomenon is difficult to predict and needs time to occur. In order to take the effects of adaptation on persistence assessments into account, the development of 'improved' RBTs should use approaches with more realistic scenarios without reducing the stringency of the test philosophy. Furthermore, researchers should not only test how different mechanisms for adaptation influence the RBT results, but also quantify their relative importance. Last but not least, further investigation of the relationship between long-term exposure in the laboratory and biodegradability is required. The use of the community systems biology approach, where multi-omics, bioinformatics and modeling tools are integrated, could improve the prediction of chemical persistency and ideally should be given appropriate consideration and incorporated into a tiered persistency assessment strategy. This approach will create a better understanding of the relationships between community composition, catabolic potential, environmental conditions and proprieties of the tested chemical. Finally, this knowledge will help to develop more accurate ready biodegradation testing and lead to a more reliable environmental risk assessment of organic chemicals.

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