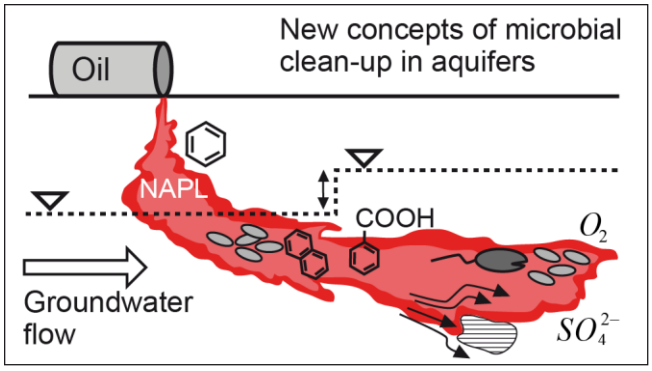
Biodegradation: Updating the concepts of control for microbial clean-up in contaminated aquifers

Abstract

Biodegradation is one of the most favored and sustainable means of removing organic pollutants from contaminated aquifers but the major steering factors are still surprisingly poorly understood. Growing evidence questions some of the established concepts for control of biodegradation. Here, we critically discuss classical concepts such as the control of biodegradation by thermodynamics and redox zonation, steady state transport scenarios, and presence of specific degrader populations. We propose updated perspectives on the controls of biodegradation in contaminant plumes: The plume fringe concept, transport limitations, as well as the effects of transient conditions on degrader populations are discussed as currently underestimated limiting factors of biodegradation processes.

Abstract Art



1. Introduction

Over the last 150 years, the number of organic chemicals released into the environment has increased dramatically [1], leaving an unprecedented chemical footprint on earth. Many groundwater contaminations result from point sources, originating from accidents or contaminations at industrial sites. These contaminations typically form plumes with high concentrations of pollutants (µg/L to mg/L range). Alternatively, chemicals may enter groundwater via dispersed application in agriculture or release from sewage treatment into rivers. Here, pesticides, pharmaceuticals, or consumer care

products are introduced as non-point source contaminants and typically occur in much smaller concentrations (ng/L to µg/L range) [2].

For what seems at first sight a daunting perspective, nature fortunately has a remedy in place: biodegradation. Microorganisms thrive on organic compounds which, at best, they oxidize to CO_2 and use to build up biomass, while at the same time electron acceptors such as molecular oxygen, nitrate, Fe(III), or sulfate are reduced (Figure 1). Alternatively, compounds may be transformed because they are reducible and serve as electron acceptors (Figure 1, right side).

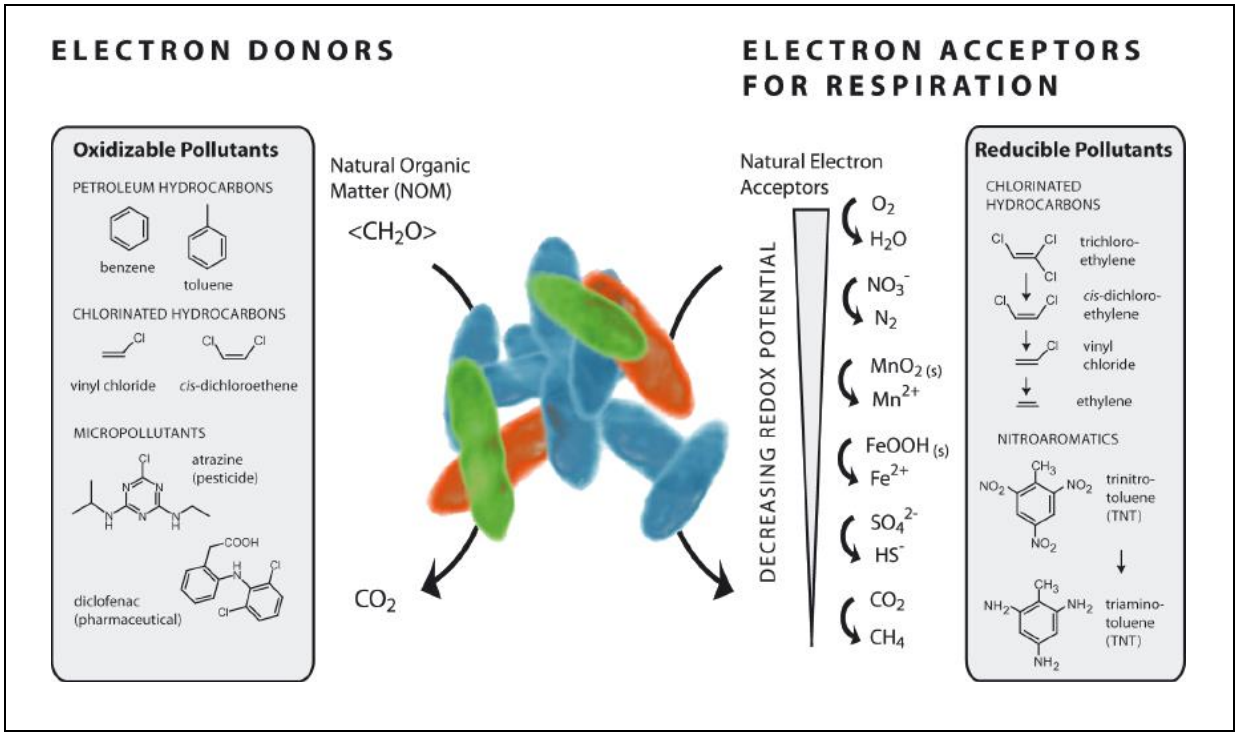


Figure 1. Contaminants can serve as electron donors or acceptors for aquifer microbes.

Despite decades of research on biodegradation and its application in bioremediation, it is still poorly understood why a given contaminant is degraded in a particular setting, or why it persists. This article revisits and challenges current concepts on the controls and limitations of biodegradation. It critically discusses (i) whether biodegradation is primarily governed by thermodynamics (i.e., redox zonation) at contaminated sites, (ii) whether biodegradation can be adequately predicted by considering the subsurface as one reactive compartment and applying terms of environmental engineering (residence time, reaction time), and (iii) the biological controls of biodegradation. We argue that groundwater ecosystems are much more heterogeneous and dynamic than currently perceived. Furthermore, we suggest that key kinetic controls of biodegradation are largely

overlooked because current concepts rely to a large part on thermodynamic considerations and steady state assumptions and processes are frequently not considered at appropriate scales. This has consequences for investigational, monitoring, management, and remediation schemes, designed to alleviate crucial controls of biodegradation.

2. Revisiting redox zonation in contaminated aquifers

In highly polluted aquifers (e.g. petroleum spills with hydrocarbon concentrations of up to 100 mg/L), an excess of dissolved electron donors (hydrocarbons) prevails over acceptors (Fig. 1). In such contaminant plumes, electron acceptors are readily depleted which is widely accepted as a major limitation of biodegradation [3, 4]. A longitudinal sequence of redox processes is assumed: methanogenic degradation close to the contaminant source, followed by sulfate reduction, manganese(IV) and iron(III)-oxide reduction, nitrate reduction, and finally aerobic processes towards the distal end of the plume [3-6] (Fig. 2 A). However, this established concept is challenged by the following considerations.

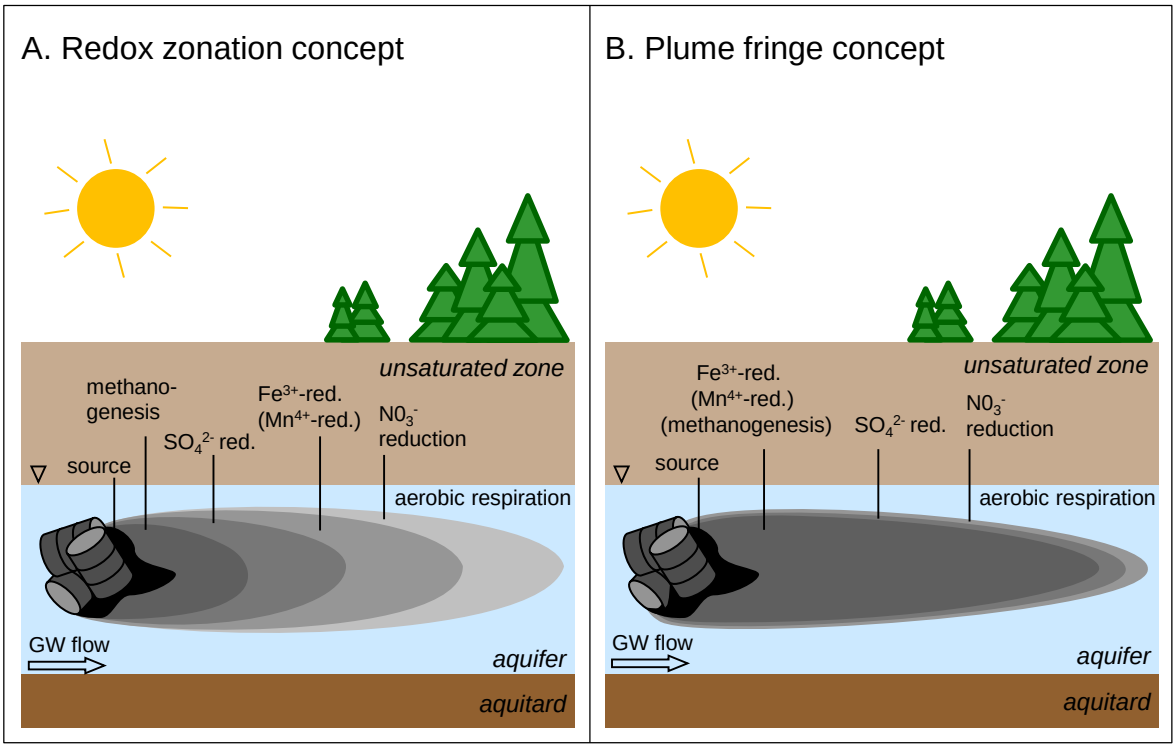


Figure 2. Comparison of the longitudinal redox zonation concept (A) and the plume fringe concept (B), both describing the spatial distribution of electron acceptors and respiration processes in a hydrocarbon contaminant plume. (B) Iron(III) reduction, manganese(IV) reduction, and methanogenesis may occur simultaneously in the core of the contaminant plume.

If dissolved electron acceptors are depleted at the head of the plume, they cannot be replenished in the downstream plume due to laminar flow and the limited transversal dispersion in porous media (Fig. 2 B). Accordingly, methanogenic degradation or reduction of mineral iron(III) phases would be the only electron accepting processes possible in the source zone or the downstream plume center. This creates an evident contradiction to the classical concept of reverse longitudinal redox zonation (Figure 2A). If dissolved electron acceptors such as sulfate or nitrate have been consumed already at the source, they cannot become available again downstream allowing for sulfate-reducing or nitrate-reducing conditions. Even if not all electron acceptors are depleted during the passage through the source zone, a downstream redox succession should develop, where first nitrate and sulfate reduction take place, followed by methanogenesis, and not *vice versa*.

In the following, we reconsider further contradictions in plume redox zonation by discussing (i) if thermodynamics truly control redox succession and microbial competition in contaminant plumes (ii) the true drivers of redox zone formation in contaminated aquifers and the importance of processes at the plume fringes, and (iii) which processes take place in the plume core.

2.1. Is thermodynamics determining microbial competition in contaminant plumes?

The theory behind every redox zonation model is that microorganisms reducing a thermodynamically more favorable electron acceptor gain more energy (Fig. 1) e.g. nitrate- vs. sulfate-reducing bacteria, [7]. In electron donor-limited systems, they should therefore be able to consume organic substrates to threshold concentrations no longer permissive for the activity of inferior respiratory guilds, which consequently become outcompeted. However, in highly contaminated aquifers, electron donors are present in excess over electron acceptors and at concentrations much higher than where competition for electron donors (i.e., available organic substrate) would be expected. Consequently, respiration processes should rather occur simultaneously as long as respective electron acceptors are present. Biodegradation activity thus becomes controlled by electron acceptor availability, rather than thermodynamics.

We propose that the reason why superior respiratory guilds such as nitrate reducers may nevertheless appear in higher abundance – even under such conditions of electron acceptor limitation – is a kinetic advantage. Gaining more energy leads to higher growth yields (*Y*) and,

therefore, growth rates (μ) (Equations 1-3; where X is the total biomass, X_0 the initial biomass, S the substrate concentration, and t the time of observation).

$$\mu = dX \div dt \times 1 \div X_0 \quad (1)$$

$$dX \div dt = -Y \times dS \div dt \quad (2)$$

$$\mu = -Y \times dS \div dt \times 1 \div X_0 \quad (3)$$

Thus, nitrate reducers can grow to higher cell numbers in a given plume compartment pretending an apparent out-competition of inferior respiratory guilds by thermodynamics. However, we propose that thermodynamic energy gain is not a major driver for redox processes in contaminated aquifers. Rather, the availability of electron acceptors at distinct spots should kinetically control biodegradation.

2.2. Importance of processes at plume fringes

In recent years, it has become apparent that biodegradation in contaminant plumes is much better explained by the 'plume fringe concept' than by the classical longitudinal redox zonation (Fig. 2) [8]. This concept is founded on the depletion of dissolved electron acceptors in the plume core. Therefore, biodegradation by oxygen, nitrate, or sulfate reduction can only be sustained at the fringes of the plume, where electron acceptors are replenished from surrounding groundwater by dispersion and diffusion ("mixing in") (Fig. 2 B) [9-11]. At an adequately high resolution of sampling, steep geochemical counter-gradients of electron donors and acceptors have indeed been observed in several contaminated aquifers thus verifying the 'plume fringe concept' [9, 12-14]. The concept also provides an appropriate explanation for the overall rather limited net biodegradation rates in hydrocarbon plumes: the poor dispersive mixing at plume fringes restricts the mass transfer of electron acceptors and, thus, limits microbial activities. Furthermore, the concept predicts that the plume fringes are the true hot spots of biodegradation *in situ* [15, 16].

The incomplete conceptual understanding of plume redox zonation brings about two fundamental caveats in many field investigations: (i) sampling at inappropriate scales and (ii) taking samples at the wrong spot [17, 18]. Thus, many studies may actually have overlooked the most relevant processes and zones of biodegradation simply because groundwater sampling was done at meter rather than at centimeter resolution. While numerous studies reported on marked differences in overall microbial community assembly along plumes [19-22], vertical heterogeneity of biodegradation activities has rarely

been considered [23-25]. Consequently, appropriately high resolution monitoring and the limitations of dispersive mixing still await a better incorporation into conceptual models and study design.

2.3. The plume core as a poorly understood compartment

Even when all dissolved electron acceptors are depleted, methanogenesis and Fe(III)- or Mn(IV)-reduction may still drive biodegradation of hydrocarbons in the plume core (Fig. 2 B) [26]. While evidence of iron-reduction has been found [27-29], methanogenic degradation seems surprisingly limited in contaminated aquifers [30]. Agitation has been shown to impede methane production in soil slurries [31], most likely by disturbing close spatial interactions between syntrophic fermenters and methanogens. In analogy, also groundwater flow may interfere with efficient interspecies electron transfer in methanogenic plume areas by flushing away hydrogen or acetate and thus the energy fluxes needed for methanogenic activity [32, 33].

3. Bottlenecks of degradation by mass transfer

The redox zonation concept is a good example of how large scale and black box approaches can miss the true controls of degradation, whereas a finer resolution better identifies the real drivers in the system. In this section, we explain that this also applies to biodegradation of micropollutants in otherwise non-contaminated aquifers where electron acceptors are normally not limiting.

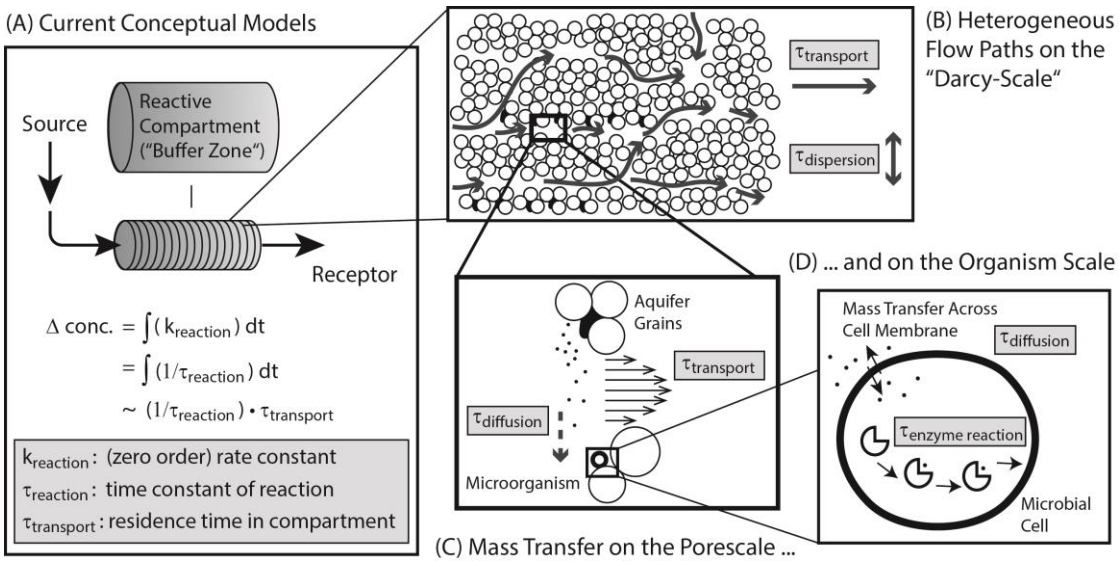


Figure 3. Plug reactor or piston flow conceptual model of contaminated aquifers where contaminant removal (time constant of reaction, $\tau_{reaction}$) is inversely proportional to average residence times $\tau_{transport}$ (A). However, heterogeneous flow paths on the "Darcy scale" (B), mass transfer on the pore scale (C), mass transfer through cell membranes on the organism scale, and

(bio)chemical enzymatic transformation on the molecular scale (D) can be important bottlenecks of biodegradation not taken into account by the simplified model.

3.1. Average residence times ignore heterogeneous flow paths and flow velocities

Current conceptual models frequently treat natural sediments and aquifers like either completely mixed or plug flow reactors (Fig. 3A) e.g., [34, 35]. In such models adopted from chemical engineering, a decrease in substrate concentration (Δ conc.) is proportional to the residence time ($\tau_{transport}$) in the reactive compartment (Fig. 3A). The potential for degradation is therefore frequently estimated from the dimensionless Damköhler number Da

$$Da = \frac{\tau_{transport}}{\tau_{reaction}} = \frac{\text{reaction rate}}{\text{transportrate}} \qquad (4)$$

where $\tau_{transport}$ is the mean residence time ("how long does it take for a compound to pass the compartment?") and $\tau_{reaction}$ the time constant of the reaction ("how long does it take for the compound to react?"). Note that the time constants are inversely correlated to the respective rates (Fig. 3A and Equation 4). If Da<<1 ($\tau_{reaction} >> \tau_{transport}$), then degradation will theoretically be incomplete or negligible; if Da>>1 ($\tau_{transport} >> \tau_{reaction}$) complete degradation can occur. This well-established concept might be a good proxy for identifying mass transfer as limiting factor in steady state systems dominated by advection. However, it is challenged by the fact that $\tau_{transport}$ and $\tau_{reaction}$ are not well defined in natural systems because they depend on multiple parameters on different scales as illustrated in Figure 3. Black box approaches which do not consider such multiple limitations will miss the opportunity for profound understanding of the steering parameters of such systems.

If transport is limiting on the Darcy scale, increased flow velocities (decreasing residence times) may affect $\tau_{reaction}$ and increase biodegradation. This at first sight counterintuitive observation may be explained when considering that increased flow velocities induce more heterogeneous flow paths by higher transversal and longitudinal dispersion [36, 37], thus increasing the apparent reaction rate by a more rapid "mixing". However, also the opposite may occur. Changes in flow velocity can create unfavorable growth conditions [37] due to changes in nutrient fluxes and redox conditions (see section 4) adversely affecting degradation (longer $\tau_{reaction}$). Average residence time alone will not give information about these ecological impacts.

3.2. Does diffusion limit bioavailability on the pore scale?

Most microorganisms are attached to sediments in groundwater [38] where diffusion may become the dominant form of substrate supply to cells at the pore scale [39-41] (Figure 3C). If supply by diffusion is slow, biodegradation is availability-limited because microorganisms consume the substrate faster than it can be delivered [41]. The apparent $\tau_{reaction}$ in a given environmental compartment is then determined by $\tau_{diffusion}$ (Figure 3C). Because diffusion to the cells takes place on the micrometer scale, steep diffusive gradients can create a situation in which much larger concentrations are observed in the surrounding water. Such limitations tend to be overlooked in conventional sampling. Whether or not such diffusion limitation is important, depends also on flow velocities.

(i) High concentrations/steady state. At high concentrations, diffusive gradients on the pore scale only build up if water flow velocities are very low [42]. Otherwise gradients form by transverse dispersion on a much larger scale corresponding to the plume-fringe concept discussed above. *(ii) Low concentrations/transient conditions.* An experimental study found that degradation rates of 2,4-dichlorphenoxyacetic acid were higher when water flow was more slowly, even though the flow path was shorter [43]. This indicates that molecules needed sufficient time to diffuse into micropores and $\tau_{transport}$ had to be sufficiently long compared to $\tau_{diffusion}$, since otherwise molecules were flushed through pore centers without degradation. *(iii) Low to medium concentrations/steady state.* If $\tau_{transport}$ is long and concentrations are low, one can expect that diffusive gradients become shallow. Consequently, also the substrate supply by diffusive fluxes becomes slower according to Fick's law. In such a case, higher flow velocities would actually be advantageous because they replenish the substrate creating steeper gradients and increasing diffusive substrate supply to the organisms. On the downside, contaminants pass through reactive zones more quickly so that reaction volumes need to be greater for efficient degradation.

3.3. Thermodynamics, mass transfer, regulation: what is limiting on the organism scale?

Micropollutants such as pesticides or pharmaceuticals are frequently detected in small concentrations (ng/L to μg/L) even when competent bacterial degraders are present [44]. This unsolved paradox of threshold concentrations on the organism scale has been explained by limitations of (i) thermodynamics, (ii) mass transfer, (iii) enzyme kinetics, and physiological reasons (the latter are discussed in section 4).

(i) Purely thermodynamic limitations for biodegradation can typically be excluded. For example at nano molar toluene concentrations, the thermodynamic driving force $\Delta_R G$ for aerobic degradation (-3890 kJ/mol) would be large enough to consume even the last toluene molecule. Biodegradation is rather kinetically limited by mass transfer to the cell as explained above. (ii) Here, substrate uptake

into the cell can be a kinetically rate-limiting step [45]. Persistence is also sometimes explained by (iii) slow biochemical transformation rates (enzyme kinetics) attributable to the intrinsically difficult-to-degrade molecular structures of xenobiotics [46]. This is supported from comparably slow degradation rates of persistent compounds even at high concentrations in batch cultures [47]. In natural systems, it has so far not been possible to distinguish the different kinds of limitation to biodegradation (in particular (ii) vs. (iii)) on the organism scale.

4. Microbial controls of biodegradation

Absence of specific degrader populations is often assumed when insufficient biodegradation is observed at a given site. Bioaugmentation (amending respective degraders) might then be applied to improve the degradation capacity [48, 49]. At organohalide-contaminated sites, bioaugmentation of microbial consortia containing e.g. *Dehalococcoides* (Dhc) strains capable of reductive dechlorination of trichloroethylene (TCE) to ethene has been successful [50]. Similarly, the effectiveness of bioaugmentation in atrazine- and MTBE-contaminated (methyl-tert-butylether) aquifers has been demonstrated [51-53]. However, even highly specialized organohalide-respiring bacteria are generally widespread in aquifers [54, 55]. Thus, it remains questionable whether respective degrader organisms were truly absent before bioaugmentation, or only present at very low abundance.

4.1. Limitation of biodegradation by microbial growth.

A substantial fraction of the microbes in aquifers is suggested to be in a status of low activity or even inactivity and dormancy [56]. Moreover, microbial communities in aquatic systems exhibit growth efficiencies (yields) much lower than known from batch cultures and chemostats, with median values below 0.3 for rivers, lakes and oceans [57, 58]. *In situ* growth rates are low (Equation 3) also for aquifer systems, and doubling times can be in the range of months to years e.g., [59]. Under optimum conditions, the presence of one degrader cell at the moment of a hydrocarbon spill would allow aerobes to form notable biomass (e.g. 10^5 to 10^6 cells per liter groundwater) within a day, while e.g. sulfate reducers or organohalide reducers (doubling time: ~10 d) may require >100 d to establish a critical population size. Indeed, a fast response has been observed for an oxic aquifer system receiving a short contaminant pulse [60], while anaerobic degradation coupled to denitirification established only over several weeks. For more recalcitrant compounds and pollutants requiring anoxic conditions for degradation (such as halogenated solvents), it might take years before reasonable numbers of degraders have developed. Thus in aquifers, also a slow community response might be misinterpreted as absence of degrader populations.

4.2. Limitations of biodegradation by microbial physiology

Total concentrations of dissolved organic carbon (DOC) in pristine groundwater are usually in the low mg L^{-1} range (0.5-2 mg L^{-1}), of which only 0.5 to 5% are readily assimilable organic carbon (AOC) [61-63]. This AOC consists of a plethora of individual compounds at extremely low individual concentrations, including organic micropollutants such as pesticides, pharmaceuticals, and many other low-level contaminants. The latter are often present below the threshold concentrations of gene induction for degradation pathways (typically in the range of 1-100 µg L^{-1}) as reported from lab experiments with single substrates [62]. In the environment, it is likely that microorganisms do not feed on only one substrate at a time. Mixed substrate utilization – where microbes can utilize a wide range of offered substrates simultaneously - has been observed in carbon-limited chemostats, which are understood to simulate close-to-natural physiological conditions [64]. This leads to much lower threshold concentrations for individual compounds implying that the degradation of one compound "helps" to degrade another compound in energetic co-metabolism. Hence, effective threshold concentrations are rather determined by the total flux of the degraded substrates [62]. Accordingly, in aquifers, the simultaneous utilization of natural carbon substrates as well as low-level pollutants by degraders likely lowers the threshold concentrations for induction, utilization, and growth [62]. This might lead to the extremely low residual concentrations observed in the environment.

Nevertheless, the research question remains how regulation of microbial metabolism works in the environment. Such knowledge might e.g. help in removing micropollutants from drinking water in engineered systems or in improving the licensing practice of pesticides [65].

4.3. Limitations of ecosystem response

Poor microbial growth rates imply that under steady state conditions where organisms can develop without disturbance, long time spans are required to establish significant biodegradation capacities. However in contaminated groundwater environments, conditions are not necessarily in steady state [66-68]. In fact, hydraulic fluctuations may represent a major control of biodegradation in groundwater by repeatedly changing the environmental conditions encountered by degraders (e.g. sudden exposure of anaerobes to oxygen). This can be exemplified by plume fringes which are characterized not only as hot spots of biodegradation activity, but also by an apparently 'specialized' degrader microbiota [23, 69] over only a few dm. However, if the prevailing geochemical conditions for microorganisms shift, degraders must continuously follow, or re-establish in other strata. Geochemical shifts of the plume could thus represent a further, as-yet unrecognized kinetic limitation of biodegradation.

Moreover, transient pulses of substrates may expose microorganisms only over insufficient time spans for degrader populations to establish. It can be speculated that under dynamic hydraulic conditions, degrader populations can never reach the biomass levels required to efficiently degrade substrate pulses. This might be one of the reasons why pesticides are so frequently encountered in aquifers. Once formed, however, degrader biomass may persist for months and perhaps even years after the source of contamination has disappeared [60 70]. Biomass established upon previous exposure events could sustain biodegradation capacities for future contaminations. Thus, aquifers may become preconditioned to efficiently degrade pollutants.

4.5. Further research needs.

So far totally overlooked is the role of grazers (protozoa) and viruses (phages) in shaping prokaryotic degrader communities and influencing *in situ* degradation rates [71]. While the influence of protozoa on bacterial community composition and *vice versa* has been shown also for contaminated groundwater habitats [72], there is contradicting evidence on either the stimulation e.g. [73, 74] or inhibition [75, 76] of biodegradation by protozoan grazing. Almost no information is available on the role of bacterio-viruses in groundwater and their influence on degrader communities and activities. Extrapolating recent advances from surface aquatic environments and marine systems, bacteriophages can be expected to play a significant role in controlling prokaryotic production and diversity. With reference to the highly specialized degrader populations found in biodegradation hot spots, ecological concepts such as the 'killing the winner' hypothesis await to be tested. It predicts that when a given population grows beyond a critical density, grazers and viruses will again decimate the population [77], which would affect biodegradation at a given site.

5. Conclusion and outlook

Several novel controls of biodegradation in contaminated aquifers have emerged in recent years and call for an update of the classical black box approaches in site assessment and restoration. Starting from the plume fringe concept, over mass transfer limitations, to the physiological properties of degraders and drivers shaping degrader community structure, this implies a need for new perspectives in groundwater research. Here, we propose that biodegradation in contaminated aquifers is mostly controlled by kinetics rather than by thermodynamics alone. Different kinetic controls are interacting in complex ways and cannot be described by flow- or residence-time-dependent degradation rates. An important reason for this is that mechanisms act on microbe to cm scale but sampling is still mostly conducted at the meter scale. To identify limiting processes, samples have to be taken at appropriately high resolution, often including intact sediment cores or

highly-resolved water sampling. Furthermore, temporal variability of processes can demand for extended monitoring campaigns with more frequent sampling intervals.

Acknowledgement: This work was funded by the EU-initial training network "Goodwater" and the Helmholtz Zentrum München.

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