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Evaluating structural robustness of a diesel-degrading bacterial consortium isolated from contaminated soil

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Abstract

It is unknown whether diesel-degrading bacterial communities are structurally and functionally robust when exposed to different hydrocarbon types. Here, we exposed a diesel-degrading consortium to model either alkanes, cycloalkanes, or aromatic hydrocarbons as carbon sources to study its structural resistance. The structural resistance of the consortium was low, with changes in relative abundances of up to four orders of magnitude, depending on hydrocarbon type and bacterial taxon. The low resistance is explained by the presence of hydrocarbon-degrading specialists in the consortium and differences in the growth kinetics on individual hydrocarbons. However, despite this low resistance, the structural and functional resilience were high, as verified by re-exposing the hydrocarbon-perturbed consortium to diesel fuel. The high resilience is either due to the short exposure time, insufficient for permanent changes in consortium structure and function, or the ability of some consortium members to be maintained during exposure on degradation intermediates produced by other members. In summary, the consortium is expected to cope with short term exposures to narrow carbon feeds while maintaining its structural and functional integrity, which remains an advantage over biodegradation approaches using single species cultures.

Keywords

biodegradation, community dynamics, hydrocarbon, robustness, resilience

1. Introduction

Selection of microbial communities for bioaugmentation of soils contaminated with hydrocarbon mixtures, such as diesel fuel, must consider their ability to adapt to temporal changes in hydrocarbon composition over the course of biodegradation [1, 2]. Similarly, if bioremediation relies on the activity of autochthonous microorganisms, temporal changes in the community structure and function can occur [3–5]. The ability of microbial communities to resist to such, potentially irreversible, changes is one of the factors determining the success of bioremediation [6]. This ability, often referred to as robustness, is usually characterized by investigating: (i) the ability of a community to resist a change in its structure after perturbation; and (ii) the potential for recovery of the community's structure to its initial state after removal of the perturbation. These two indicators of structural robustness are referred to with the terms *structural* resistance and *structural* resilience, respectively [7, 8]. The structure of a community may also influence its *functional* resilience, understood as the ability of a community to maintain a particular activity despite perturbation [7, 9, 10].

Vila et al (2010) showed that successive biodegradation of particular hydrocarbon fractions in the marine environment is conducted by different, temporally dominant bacterial taxa [11]. Also Kostka et al. (2011) showed that *Alcanivorax* was the dominant taxon during linear and branched alkanes utilization in the early stages of crude oil biodegradation in marine environment, whereas *Acinetobacter*, *Marinobacter* and *Pseudomonas*, identified as both alkane and aromatics degraders, were the most abundant at the later stage of biodegradation [2]. Diesel-degrading consortia are similarly not thought to consists of generalist bacteria with ability for growth on all major

hydrocarbon types (that is, linear and branched alkanes, cycloalkanes and aromatic hydrocarbons) present in a petroleum diesel fuel [1]. It is expected that the structural resistance of diesel-degrading consortia is low. If a given hydrocarbon (e.g., the branched-chain alkane pristane) is utilized by one consortium member only, the change in structure of the consortium, when exposed to that hydrocarbon, will be governed by the initial fraction of cells belonging to that consortium member, the kinetics of growth of the degrading member on that hydrocarbon, and the exposure time to the hydrocarbon. On the other hand, if a hydrocarbon can be utilized by many members of the consortium, its change in structure will depend mainly on the differences in the kinetics of growth between consortium members on that hydrocarbon. Both exposure time and the kinetics of growth are expected to play a key role in determining the structural resilience: the ability of the perturbed community to recover its initial state.

Resistance and resilience of microbial communities must be considered when constructing consortia for bioaugmentation of hydrocarbon mixtures [6]. Low resistance is undesirable if a consortium isolated on a specific hydrocarbon mixture, e.g. petroleum diesel fuel, is exposed to various hydrocarbons present in the diesel fuel over the course of biodegradation, unless the consortium is structurally and functionally resilient. Ideally, the consortium should be able to cope with narrow carbon feeds and adapt readily to varying composition of a hydrocarbon mixture over time. To date, there is limited knowledge on the structural resistance and resilience of diesel-degrading bacterial consortia associated with exposure to different hydrocarbon types.

In this paper, we use a diesel-degrading bacterial consortium to evaluate its: (i) *structural resistance*, measured as the degree of change in structure in terms of abundance of the dominant bacterial taxa when deprived of its typical energy source (i.e., petroleum diesel fuel) and perturbed to grow on individual model aliphatic, cycloaliphatic, or aromatic hydrocarbons; (ii) *structural resilience*, measured as the degree of recovery of the perturbed consortium to its initial state when re-exposed to diesel fuel after its perturbation; and (iii) *functional resilience*, measured as the ability of the previously perturbed and re-exposed consortium to mineralize the diesel fuel. The relative abundance of seven core taxa, used to describe the structure of the perturbed and recovered communities, was quantified using real-time PCR and the ddCt method for relative quantification [12]. In total, 6 aliphatic (*n*-dodecane, *n*-hexadecane, *n*-octadecane, *n*-docosane, heptamethylnonane, pristane), 5 cycloaliphatic (decalin, cycloheptane, ethylcyclohexane, butylcyclohexane, bicyclohexyl), and 8 aromatic (acenaphthene, ethylbenzene, 1,5-dimethyltetraline, o-xylene, cyclohexylbenzene, naphthalene, 2-ethylnaphthalene, phenanthrene) hydrocarbons, which represent major hydrocarbon types present in petroleum diesel fuel, were employed. In addition, biodiesel (a mixture of fatty acid methyl esters) derived from rapeseed, was used. Thereby, we show how the overall low structural resistance depends on the type of hydrocarbon and bacterial taxon, and how the perturbed consortium recovers its initial state, presenting high structural and functional resilience.

2. Materials and methods

2.1. Bacterial consortium

The bacterial community employed in this study had been isolated from a soil contaminated with crude oil using selective enrichments with diesel fuel as source of carbon and energy [13]. The community contained bacteria of the following taxa: *Achromobacter* sp. (AchrP), *Alcaligenes* sp. (AlcP), *Citrobacter* sp. (CKK), *Comamonadaceae* (ComP), *Sphingobacterium* sp. (SphiP), *Pseudomonas* sp. (PseuP), and *Variovorax* sp. (VariP) [14]. The community has a degradation potential toward diesel and biodiesel fuels [13–16], and is able to mineralize all the individual hydrocarbons employed in this study [17].

The community was stored in 30% (v/v) glycerol stocks at -80°C. To prepare an inoculum, a stock suspension (1 mL) was transferred to a 300 mL Erlenmeyer flask containing 50 mL of mineral medium [13] and petroleum diesel fuel (0.5%, v/v), and was cultivated for 24 h at 25°C on an orbital shaker (120 rpm). Then, a 1 mL aliquot of the cell suspension was transferred to a new enrichment flask and the culture was grown for 3 days in the same conditions. This step was repeated three times and cells from the last enrichment were centrifuged at 10,000×g, washed twice with 40 mL of the mineral medium, re-suspended in the medium, and used as inoculum. This inoculum is further referred to as the initial community.

2.2. Hydrocarbons

To study the structural resistance of the community against carbon source changes, a total of 19 individual hydrocarbons representing all major hydrocarbon types present in petroleum diesel fuel, were used (Table 1). Structural resistance was also assessed against 4 model hydrocarbon mixtures and against biodiesel derived from rapeseed (Table 1). The hydrocarbons were purchased from Sigma Aldrich. The biodiesel, produced according to DIN E 51606 [18] was purchased from a supplier in Germany, whereas petroleum diesel fuel, produced according to EN 590:2004 [19] was purchased from a petrol station (PKN Orlen, Poland). Prior to experiments, all the fuels had been sterilized by filtration (Millex, pore size of 0.2 μm, Millipore).

2.3. Repeated exposure to hydrocarbons

The experimental design is presented in Figure 1. The structural resistance of the community was evaluated by comparing the relative abundances of core taxa within the initial community (that is, the preculture grown on diesel fuel) with that of the communities perturbed by the growth on individual or mixtures of defined hydrocarbons (Table 1) as sources of carbon in repeated growth experiments. First, cells (1-mL cell suspension) were transferred from the inoculum culture to 500-mL bottles containing 50 mL medium and hydrocarbon or hydrocarbon mixture (including diesel fuel as initial carbon source) at concentrations given in Table 1 and cultivated at 25°C at 120 rpm for 7 days. Then, aliquots were transferred to new set of bottles containing medium (the ratio of inoculum volume to total liquid volume was 1:50) and same hydrocarbons at their respective concentrations and cultivated in the same conditions for 7 days. This step was repeated 3 times, reaching 5 steps in total.

The structural resilience of the community was evaluated by comparing the structure of the initial community with that of the communities first perturbed by growth on individual hydrocarbons or defined mixtures, as described above, and then returned to grow on complex petroleum diesel fuel as sole carbon source. Again, growth on diesel was repeated by 2 sequential

dilution passages (again, the ratio of inoculum volume to total liquid volume was 1:50), reaching 3 steps in total. Aliquots from the last growth passage were sampled to determine community structure and are referred to as recovered communities.

To assess the functional community resilience, we compared the initial and recovered communities with respect to the mineralization kinetics of petroleum diesel fuel in saturated sand microcosms.

2.4. Structure of the community

Real-time PCR and the ddCt method for relative quantification (12), employed earlier by Cyplik et al. (2011) [14], were used to quantify the relative abundance of the core taxa, from which we described the structure of the perturbed and recovered communities. In the ddCt methods, the relative abundance is expressed as relative quantity (RQ), where the amount of target rRNA genes for the seven bacterial taxa retrieved from the studied communities is normalized to the total number of bacterial rRNA in the respective community (Eq. 1).

$$RQ = \frac{\left(\frac{q^T_{t(x)}}{q^B_{t(x)}}\right)}{\left(\frac{q^T_{t(0)}}{q^B_{t(0)}}\right)} = \frac{X_{N,q}}{X_{N,cb}} = (1+E)^{-(\Delta C_{T,q} - \Delta C_{T,cb})} = (1+E)^{-\Delta\Delta C_T} = 2^{-\Delta\Delta C_T} \qquad \text{Eq. 1.}$$

where q^T_t is the quantity of a taxon of interest (i.e., target in ddCt terms) in any perturbed or recovered community (i.e., sample) at time *t* equal to 5 and 8 weeks for the perturbed and recovered communities, respectively; q^B_t is the quantity of total bacteria (i.e., reference) in the sample at time *t*; q^T_0 is the quantity of the target in the initial community (i.e. calibrator) at time *t* equal to zero; q^B_0 is the quantity of the reference in the initial community at time *t* equal to zero. $X_{N,q}$ and $X_{N,cb}$ are thus the normalized amount of the target taxon in the sample and the normalized amount of the target taxon in the initial community, respectively. In the ddCt method, the *RQ* is computed from the difference in threshold cycles for the target and the reference in a sample ($\Delta C_{T,q}$) and the difference in threshold cycles for target and reference in a calibrator ($\Delta C_{T,cb}$). The efficiency of the target (*E*) was assumed equal to 1 [12].

Biomass was collected by centrifugation of the liquid culture at 8228×g for 15 min. Total DNA was extracted and purified using Genomic Mini kit (A&A Biotechnology, Poland) following the manufacturer`s instruction with initial pretreatment with lysozyme (45 mg/mL), lysostaphin (200 U/mL) and mutanolysin (250 U/mL). The characteristics of primers and probe sets for the PCR can be found in Cyplik et al. (2011) [14].

2.5. Mineralization kinetics of diesel fuel in saturated sand microcosms

Mineralization of diesel fuel was studied in saturated sand microcosms, as described in Lisiecki et al. (2014) [20]. Briefly, 50 g of dry sand was placed in a sealed 1-litre glass bottles. The microcosms were spiked with diesel fuel (16 g/kg dry sand) applied on the sand surface. Then, the microcosms were inoculated with the initial community, or with the recovered community (re-

exposed to diesel fuel after exposure to hydrocarbons) by applying a dense cell suspension (1 mL; OD_{600nm} 3±0.1) on the sand surface. Afterwards, 14 mL of mineral medium was added to obtain full saturation. Microcosms were maintained without disturbance at 20°C for 28 days. The mineralization was determined by measuring CO_2 content in a base trap (10 mL of 0.75 M NaOH in a 20 mL vial) placed in microcosms. Titration of the diluted NaOH and Na_2CO_3 solution from the trap with 0.1 M HCl was done using an automatic titrator (Metrohm titroprocessor 686). Each experiment was carried out in triplicates.

3. Results

The response of the studied consortium to model hydrocarbons was hydrocarbon- and taxon-specific (Fig 2, left panel). Both increases and decreases in relative taxon abundance, up to four orders of magnitude relative to the initial community, were observed.

When exposed to *n*-alkanes, the largest changes in abundance were found for *Citrobacter* sp. (an increase of four orders of magnitude), and *Achromobacter* sp. (a decrease of three orders of magnitude) (Fig 2a). The response for other community members was somewhat smaller, within one order of magnitude. For branched-alkanes, the *Alcaligenes* sp., *Achromobacter* sp., *Citrobacter* sp., *Comamonadaceae and Pseudomonas* sp. taxa increased in relative abundance up to three orders of magnitude after exposure to heptamethylnonane and pristane (Fig 2a). On the other hand, *Sphingobacterium* sp. decreased in relative abundance after exposure to branched alkanes, especially pristane. No significant changes were observed for *Variovorax* sp. For cycloalkanes, an increase in abundance of up to two orders of magnitude (*Achromobacter, Comamonadaceae and Variovorax* sp.) was observed, while *Citrobacter* sp. and *Sphingobacterium* sp. did not significantly change in their abundance (Fig. 2b). For aromatic hydrocarbons, *Alcaligenes* sp., *Comamonadaceae*, *Pseudomonas* sp., *Sphingobacterium* sp. *and Variovorax* sp. were, in most cases, up to four orders of magnitude more abundant in comparison to their relative quantity in initial community, whereas the abundance of *Achromobacter* sp. and *Citrobacter* sp. decreased up to three orders of magnitude (Fig. 2c).

Overall, these results suggest that the structural resistance of the consortium was low. However, when these hydrocarbon-perturbed cultures were re-exposed to diesel fuel, the relative abundance of the dominant taxa returned close to the values in the initial community (Fig. 2e-h). The RQ values (log_{10}-transformed) ranged from -0.5 to 0.5. Further, in the 28-day mineralization kinetics test, all recovered communities showed similar kinetics of diesel mineralization (Fig. 2i-l). This suggests that the ability to degrade diesel fuel, did not change, and functional resilience was high.

4. Discussion

4.1. Explaining low structural resistance and high resilience

Structural changes in the community are expected when deprived of its normal energy source, the diesel fuel, and forced to survive on a single hydrocarbon. Allison and Martiny (2008) already

showed that the composition of microbial communities is sensitive to changes in various carbon amendments, including petroleum [21]. Although biodegradation of individual hydrocarbons was not verified in the present study, the consortium did have a potential to mineralize all the studied hydrocarbons when supplied as a mixture [17], suggesting that each individual hydrocarbon was degraded by one or more community members also when supplied as sole source of carbon and energy. This is further confirmed by an increase in turbidity that was observed in the flasks due to cell growth.

Hydrocarbon toxicity is not likely to have influenced the community structure as the consortium had been adapted to relatively high (>5 mg/L) concentration of diesel fuel [13], and individual hydrocarbons were applied at subinhibitory levels. Thus, an increase of RQ values of a taxon when exposed to a specific hydrocarbon can indicate that either: (i) the hydrocarbon was a primary carbon and energy source for that taxon, or (ii) the hydrocarbon was not a primary carbon and energy source for that taxon but the taxon benefited from its biodegradation by another community member(s). On the other hand, a decrease in relative abundance could indicate that either: (i) a taxon did not have the ability to grow on the hydrocarbon and did not benefit from its biodegradation by other member(s), or (ii) the taxon degraded the hydrocarbon but its specific growth rate was smaller compared to other members of the consortium.

Allison and Martiny (2008) similarly showed that the composition of microbial communities does not recover for some time after disturbance [21]. Although we did not evaluate the functional resistance of the studied bacterial community during the study, a significant changes in mineralization of diesel fuel during stress (passages on single hydrocarbons) are expected to occur. The soil microbial community structure and functions can be both positively or negatively correlated depending on the used perturbation and measured function [22]. However, the mineralization of petroleum hydrocarbons (or hydrocarbon mixtures) seem to change with changing microbial community structure, since not all microorganisms present in the environmental communities can degrade all available carbon sources [1, 23]. Hamamura et al. (2013) already showed that mineralization of ^{14}C-hexadecane was different among the same soil samples with diverse microbial community structures (induced by the contamination of soil with different hydrocarbon mixtures) [5]. In our study, despite the apparently low structural resistance, the structural and functional resilience were relatively high. This may suggest that either: (i) each identified consortium member was able to grow on each studied hydrocarbon, albeit at various rates; or (ii) not each consortium member was able to grow on each studied hydrocarbon, but the exposure time was short enough to avoid irreversible changes in community structure. The latter explanation is more likely as in diesel-degrading consortia, it is known that some bacteria degrade a wide variety of hydrocarbons (and are therefore generalists), while others are specialized to few compounds (and are therefore specialists) [1].

4.2. Hydrocarbon-degrading specialist and generalists in the consortium

Specialists are likely found in taxa that displayed the highest difference in relative abundance between individual hydrocarbons, such as seen for some branched-chain alkane or aromatic hydrocarbon exposures.

Linear alkanes are generally easier to degrade as compared to the branched ones [24–27]. In our consortium, however, *Sphingobacterium* sp. was the only alkane-degrading taxon that had decreased RQ values when exposed to branched alkanes. By contrast, *Achromobacter* sp. increased in relative abundance when exposed to branched alkanes only (when exposed to *n*-alkanes a decrease in relative abundance was observed). Thus, *Sphingobacterium* sp. could be dominated by strains which have alkane oxidation mechanisms specific to *n*-alkanes, such as β-oxidation, whereas *Achromobacter* sp. could be dominated by bacteria which have alkane oxidation mechanisms specific to branched alkanes, such as ω-oxidation [28]. Another example of specialized alkane-degraders could be strains within the *Citrobacter* sp. taxon, which increased significantly in relative abundance when exposed to *n*-alkanes or biodiesel, but less when exposed to branched alkanes and cycloalkanes, and decreased in relative abundance when exposed to all aromatic hydrocarbons. This is supported by the fact that *Citrobacter* sp. showed the highest increase in relative abundance among all taxa when exposed to biodiesel, which was expected as biodegradation of fatty acid methyl esters from biodiesel proceeds through the pathway known for *n*-alkanes (i.e. through fatty acid intermediates [26]). This is also in agreement with the ability of *n*-alkane degraders to grow on the *n*-alkane oxidation products [29].

Metabolic pathways of cycloalkanes are less characterized than those for linear or branched alkanes [26, 30]. During oxidation of a cyclic alkanes dicarboxylic acids are usually formed, similarly to ω-oxidation of branched alkanes [28, 30]. This could explain why the species expected to be primary *n*-alkane degraders, such as *Citrobacter* sp. or *Sphingobacterium* sp. did not increase in abundance when exposed to cyclic alkanes. By contrast, based on RQ values *Alcaligenes* sp., *Comamonadaceae* are expected to be dominated by generalists with regard to their potential for degradation of alkanes, with both β-oxidation and ω-oxidation mechanisms co-occurring within these taxa [31].

Apart from *Achromobacter* sp. and *Citrobacter* sp., all taxa increased in relative abundance when exposed to aromatic hydrocarbons. This is consistent with the ability of AlcP, ComP, PseuP SphiP and VariP to degrade various aromatic hydrocarbons [32–41]. Relatively large increases in abundance after exposure to aromatic hydrocarbons are associated with somewhat lower increase in abundance of the taxa when exposed to *n*-alkanes, indicating that aromatic hydrocarbons are the preferential carbon source within the studied community. However, bacteria belonging to *Pseudomonas* sp. and *Alcaligenes* sp. are known to degrade a wide variety of compounds, including alkanes (e.g. dodecade, pristine) [37, 42, 43], cycloalkanes (e.g. cyclohexane, decaline) [37, 44, 45] and aromatic hydrocarbons (e.g. benzene, phenanthrene) [35, 36, 37, 39, 40] and are thus expected to be hydrocarbon-degrading generalists.

5. Conclusions

We showed that a diesel-degrading bacterial consortium was structurally and functionally robust when employed for biodegradation of various hydrocarbons. The robustness of the microbial community was evaluated by investigating the structural and functional resilience and resistance. Despite low structural resistance, which was explained by the presence of hydrocarbon-degrading specialists in the consortium and differences in the kinetics of growth, the structural and functional

resilience were high. The robustness of the diesel-degrading consortium is an advantage when employed for biodegradation (e.g. bioaugmentation) of environments which may have varying hydrocarbon composition over time. Such a consortium is expected to be able to cope with narrow carbon feeds yet maintaining structural and functional integrity, which is advantageous over biodegradation carried out by single species.

Our findings raise several additional questions. First, it is unknown whether the results are applicable to other hydrocarbon-degrading consortia isolated on complex hydrocarbon mixtures. Second, it is unknown whether the structural and functional robustness is a property of consortia isolated from contaminated environments, or whether such a (robust) consortium can be constructed from single species of known ability to degrade specific hydrocarbons. Third, the applicability of these results to field conditions needs to be examined as mass transfer limitations of carbon sources and availability of nutrients may play a large role in shaping community structure. Finally, it is unknown whether the consortium maintains its structural and functional integrity if longer exposure times are used. Biodegradation time scales in soils or aquifers are longer than a few weeks, in which case structural robustness and functional performance might be challenged.

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Figure captions

Fig. 1. Experimental design for evaluating structural resistance and resilience and functional resilience of a diesel-degrading bacterial consortium.

Fig 2. Relative quantity (RQ) values (in $\log_{10}$ scale) of hydrocarbon-perturbed cultures of the diesel-degrading bacterial consortium (a-d); of the hydrocarbon-perturbed cultures re-exposed to diesel fuel (e-h), and diesel fuel mineralization kinetics with respect to recovered communities and initial community (i-l). Error bars represent standard errors of the mean.

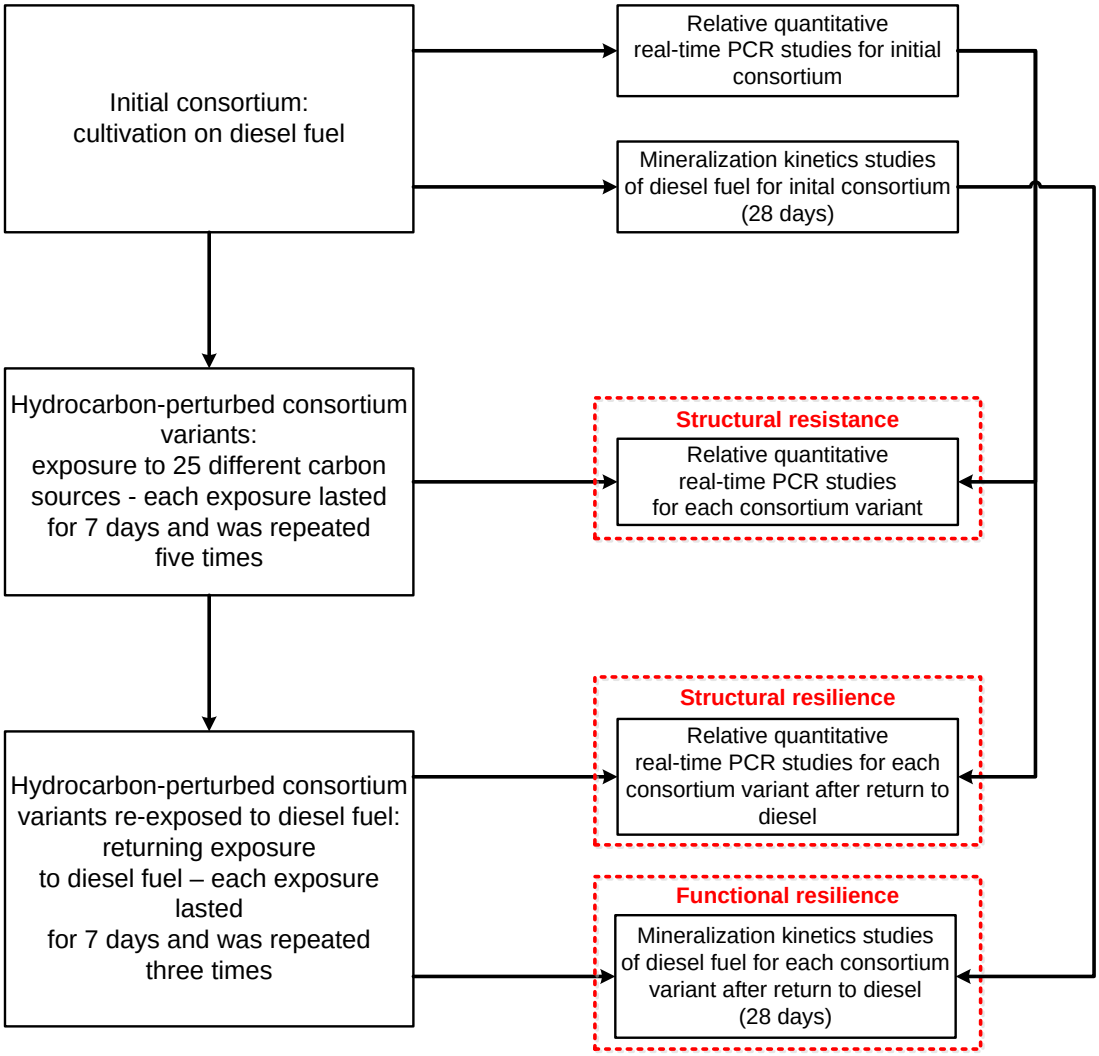
Initial consortium:
cultivation on diesel fuel
Relative quantitative
real-time PCR studies for initial
consortium
Mineralization kinetics studies
of diesel fuel for inital consortium
(28 days)
Hydrocarbon-perturbed consortium
variants:
exposure to 25 different carbon
sources - each exposure lasted
for 7 days and was repeated
five times
Structural resistance
Relative quantitative
real-time PCR studies
for each consortium variant
Structural resilience
Relative quantitative
real-time PCR studies for each
consortium variant after return to
diesel
Hydrocarbon-perturbed consortium
variants re-exposed to diesel fuel:
returning exposure
to diesel fuel – each exposure
lasted
for 7 days and was repeated
three times
Functional resilience
Mineralization kinetics studies
of diesel fuel for each consortium
variant after return to diesel
(28 days)

Fig. 1.

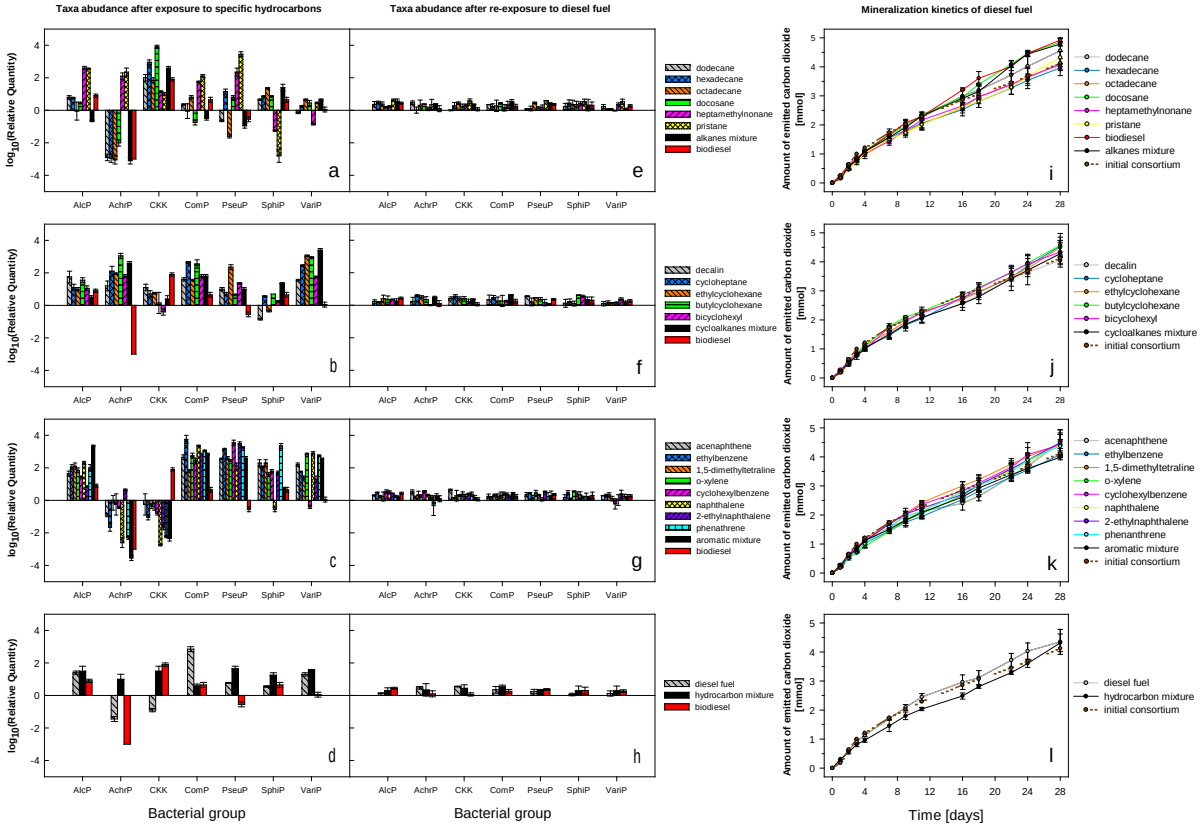
Taxa abudance after exposure to specific hydrocarbons
Taxa abudance after re-exposure to diesel fuel
Mineralization kinetics of diesel fuel
log10(Relative Quantity)
AlcP
AchrP
CKK
ComP
PseuP
SphiP
VarlP
Bacterial group
Amount of emitted carbon dioxide [mmol]
Time [days]
a
b
c
d
e
f
g
h
i
j
k
l
dodecane
hexadecane
octadecane
docosane
heptamethylnonane
pristane
alkanes mixture
biodiesel
initial consortium
decalin
cycloheptane
ethylcyclohexane
butylcyclohexane
bicyclohexyl
cycloalkanes mixture
acenaphthene
ethylbenzene
1,5-dimethyltetraline
o-xylene
cyclohexylbenzene
naphthalene
2-ethylnaphthalene
phenanthrene
aromatic mixture
diesel fuel
hydrocarbon mixture

Fig. 2.