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

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38 **Abstract**

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

53

54 **Keywords**

55 biodegradation, community dynamics, hydrocarbon, robustness, resilience

56

57 **1. Introduction**

58

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

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

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

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

98 In this paper, we use a diesel-degrading bacterial consortium to evaluate its: (i) *structural*
99 *resistance*, measured as the degree of change in structure in terms of abundance of the dominant
100 bacterial taxa when deprived of its typical energy source (i.e., petroleum diesel fuel) and perturbed
101 to grow on individual model aliphatic, cycloaliphatic, or aromatic hydrocarbons; (ii) *structural*
102 *resilience*, measured as the degree of recovery of the perturbed consortium to its initial state when
103 re-exposed to diesel fuel after its perturbation; and (iii) *functional resilience*, measured as the ability
104 of the previously perturbed and re-exposed consortium to mineralize the diesel fuel. The relative
105 abundance of seven core taxa, used to describe the structure of the perturbed and recovered
106 communities, was quantified using real-time PCR and the ddCt method for relative quantification
107 [12]. In total, 6 aliphatic (*n*-dodecane, *n*-hexadecane, *n*-octadecane, *n*-docosane,
108 heptamethylnonane, pristane), 5 cycloaliphatic (decalin, cycloheptane, ethylcyclohexane,
109 butylcyclohexane, bicyclohexyl), and 8 aromatic (acenaphthene, ethylbenzene, 1,5-
110 dimethyltetraline, *o*-xylene, cyclohexylbenzene, naphthalene, 2-ethylnaphthalene, phenanthrene)
111 hydrocarbons, which represent major hydrocarbon types present in petroleum diesel fuel, were
112 employed. In addition, biodiesel (a mixture of fatty acid methyl esters) derived from rapeseed, was
113 used. Thereby, we show how the overall low structural resistance depends on the type of
114 hydrocarbon and bacterial taxon, and how the perturbed consortium recovers its initial state,
115 presenting high structural and functional resilience.

117 **2. Materials and methods**

118 119 **2.1. Bacterial consortium**

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

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

135

136 **2.2. Hydrocarbons**

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

145

146 **2.3. Repeated exposure to hydrocarbons**

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

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

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

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

169 **2.4. Structure of the community**

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

$$177 \quad RQ = \frac{\left(\frac{q_{t(x)}^T}{q_{t(x)}^B} \right)}{\left(\frac{q_{t(0)}^T}{q_{t(0)}^B} \right)} = \frac{X_{N,q}}{X_{N,cb}} = (1 + E)^{-(\Delta C_{T,q} - \Delta C_{T,cb})} = (1 + E)^{-\Delta \Delta C_T} = 2^{-\Delta \Delta C_T} \quad \text{Eq. 1.}$$

178 where q_t^T is the quantity of a taxon of interest (i.e., target in ddCt terms) in any perturbed or
 179 recovered community (i.e., sample) at time t equal to 5 and 8 weeks for the perturbed and recovered
 180 communities, respectively; q_t^B is the quantity of total bacteria (i.e., reference) in the sample at time
 181 t ; q_0^T is the quantity of the target in the initial community (i.e. calibrator) at time t equal to zero; q_0^B
 182 is the quantity of the reference in the initial community at time t equal to zero. $X_{N,q}$ and $X_{N,cb}$ are
 183 thus the normalized amount of the target taxon in the sample and the normalized amount of the
 184 target taxon in the initial community, respectively. In the ddCt method, the RQ is computed from
 185 the difference in threshold cycles for the target and the reference in a sample ($\Delta C_{T,q}$) and the
 186 difference in threshold cycles for target and reference in a calibrator ($\Delta C_{T,cb}$). The efficiency of the
 187 target (E) was assumed equal to 1 [12].

189 Biomass was collected by centrifugation of the liquid culture at 8228×g for 15 min. Total
 190 DNA was extracted and purified using Genomic Mini kit (A&A Biotechnology, Poland) following
 191 the manufacturer`s instruction with initial pretreatment with lysozyme (45 mg/mL), lysostaphin
 192 (200 U/mL) and mutanolysin (250 U/mL). The characteristics of primers and probe sets for the PCR
 193 can be found in Cyplik et al. (2011) [14].

195 **2.5. Mineralization kinetics of diesel fuel in saturated sand microcosms**

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

200 exposed to diesel fuel after exposure to hydrocarbons) by applying a dense cell suspension (1 mL;
201 $OD_{600nm} 3\pm 0.1$) on the sand surface. Afterwards, 14 mL of mineral medium was added to obtain full
202 saturation. Microcosms were maintained without disturbance at 20°C for 28 days. The
203 mineralization was determined by measuring CO₂ content in a base trap (10 mL of 0.75 M NaOH in
204 a 20 mL vial) placed in microcosms. Titration of the diluted NaOH and Na₂CO₃ solution from the
205 trap with 0.1 M HCl was done using an automatic titrator (Metrohm titroprocessor 686). Each
206 experiment was carried out in triplicates.

207

208 **3. Results**

209

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

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

228

229 Overall, these results suggest that the structural resistance of the consortium was low.
230 However, when these hydrocarbon-perturbed cultures were re-exposed to diesel fuel, the relative
231 abundance of the dominant taxa returned close to the values in the initial community (Fig. 2e-h).
232 The RQ values (log₁₀-transformed) ranged from -0.5 to 0.5. Further, in the 28-day mineralization
233 kinetics test, all recovered communities showed similar kinetics of diesel mineralization (Fig. 2i-l).
234 This suggests that the ability to degrade diesel fuel, did not change, and functional resilience was
235 high.

235

236 **4. Discussion**

237

238 **4.1. Explaining low structural resistance and high resilience**

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

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

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

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

277 278 **4.2. Hydrocarbon-degrading specialist and generalists in the consortium**

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

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

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

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

316

317 **5. Conclusions**

318

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

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

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

339

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341

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346

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509 **Figure captions**

510

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

513

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

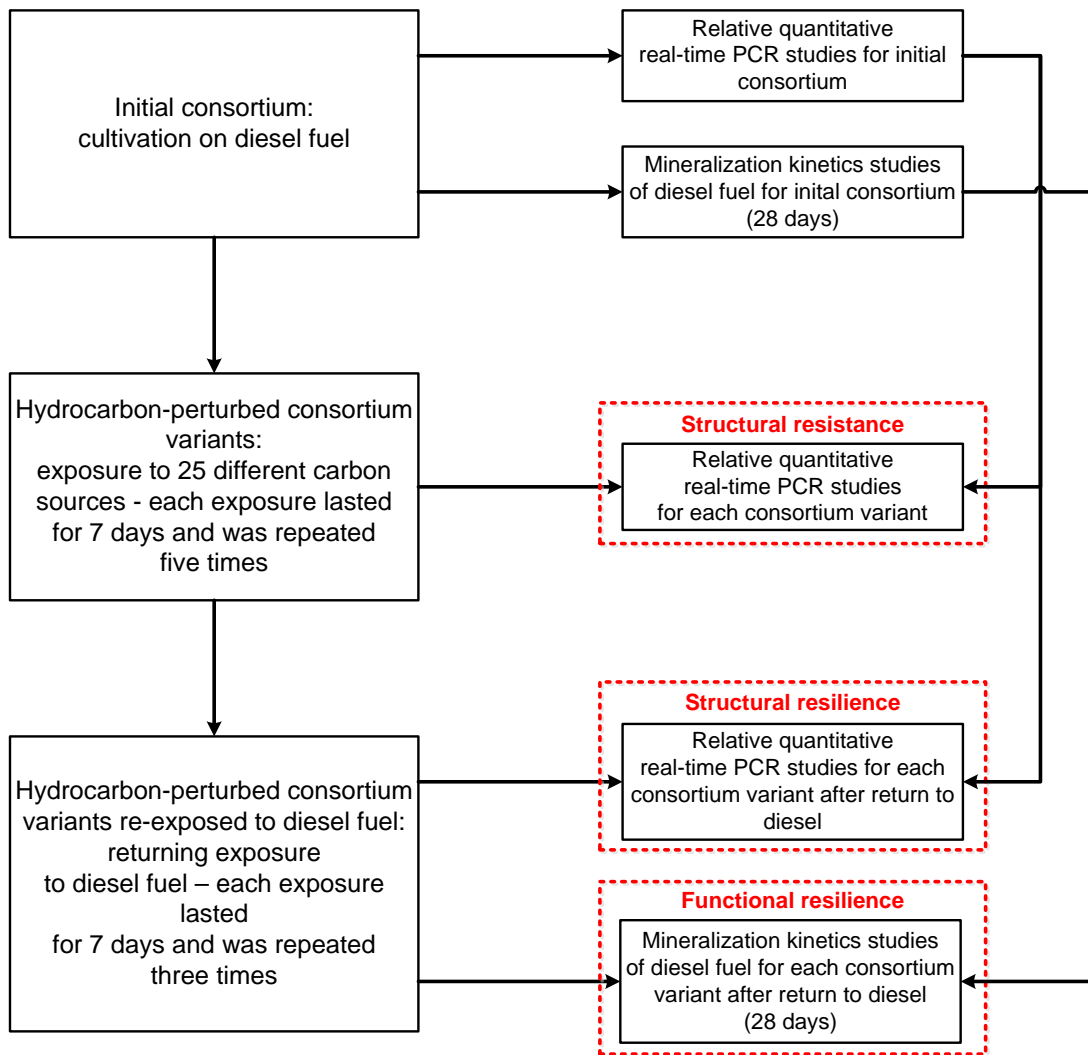


Fig. 1.

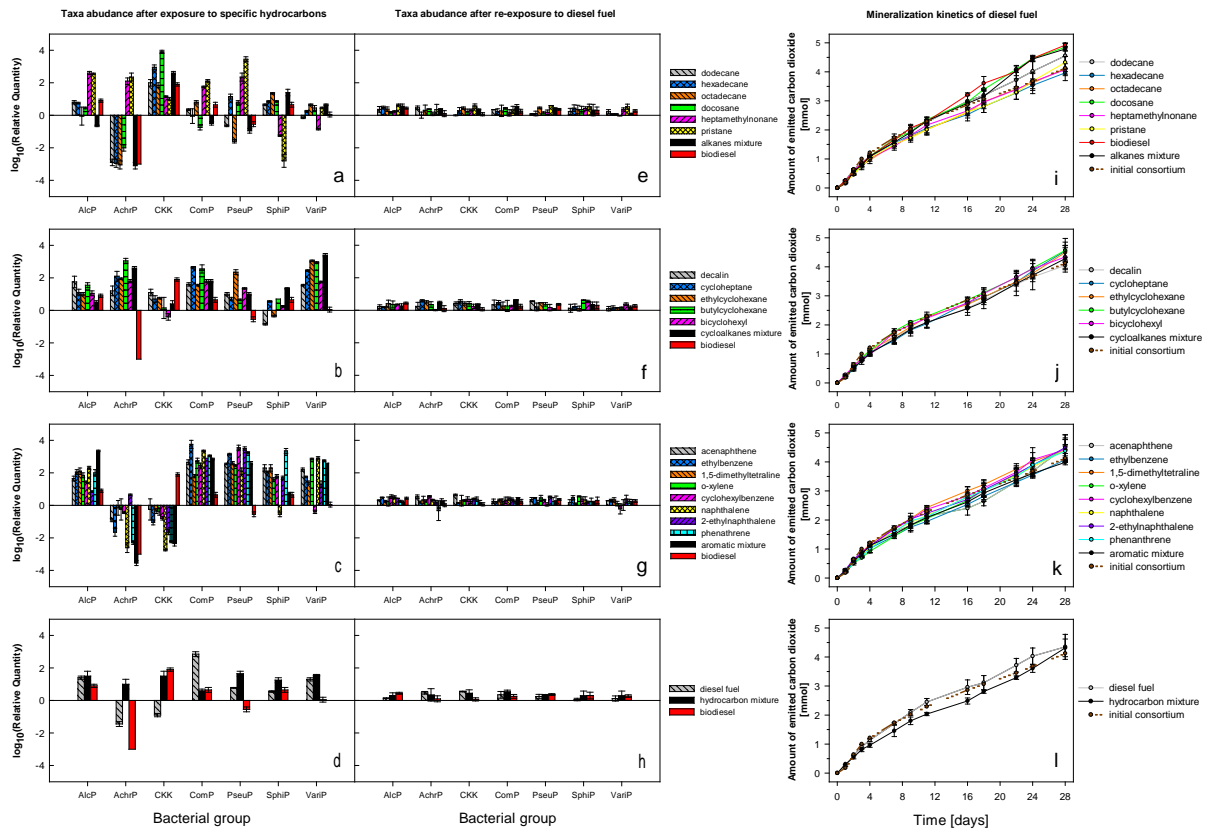


Fig. 2.