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Stable Isotope Switching (SIS): A New Stable Isotope Probing (SIP) Approach to Determine Carbon Flow in the Soil Food Web and Dynamics in Organic Matter Pools

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Running head: ^{13}C Stable Isotope Switching (SIS)

Abstract

Rationale: Recent advances in stable isotope probing (SIP) have allowed direct linkage of microbial population structure and function. This paper details a new development of SIP, Stable Isotope Switching (SIS), which allows the simultaneous assessment of C uptake, turnover and decay, and the elucidation of soil food webs within complex soils or sedimentary matrices.

Methods: SIS utilises a stable isotope labelling approach whereby the ^{13}C-labelled substrate is switched part way through the incubation to a natural abundance substrate. A $^{13}CH_4$ SIS study of landfill cover soils from Odcombe (Somerset, UK) was conducted. C assimilation and dissimilation processes were monitored through bulk elemental analysis-isotope ratio mass spectrometry and compound specific gas chromatography-combustion-isotope ratio mass spectrometry targeting a wide range of biomolecular components including: lipids, proteins and carbohydrates.

Results: Carbon assimilation by primary consumers (methanotrophs) and sequential transport into secondary (Gram negative and positive bacteria) and tertiary consumers (Eukaryotes) was observed. Up to 45% of bacterial membrane lipid C was determined to be directly derived from CH_4 and at the conclusion of the experiment ca. 50% of bulk soil C derived directly from CH_4 was retained within the soil.

Conclusions: This is the first estimate of soil organic carbon derived from CH_4 and is comparable to levels observed in lakes that have high levels of benthic methanogenesis. SIS opens the way for a new generation of SIP studies aimed at elucidating total C dynamics (incorporation, turnover and decay) at the molecular level in a wide range of complex environmental and biological matrices.

Introduction

A range of new techniques, have recently emerged to study environmental microorganisms *in situ* without the need to establish laboratory cultures.[1] Among these so-called '*culture independent* methods' is stable isotope probing (SIP), an approach which involves the incubation of environmental soils or sediments with stable isotope labelled substrates. In

45 many cases a simple 'pulse chasing' approach is utilised, whereby a short application or
46 single dose of a highly enriched ^{13}C-labelled substrate is applied to complex environmental
47 samples and traced to identify the fate of the substrate. Subsequently, the metabolic activities
48 of microorganisms are assessed through determination of label incorporation into
49 biochemical components of cells of active members of the microbial population including
50 DNA,[4] RNA[5] and phospholipid fatty acids (PLFAs).[1] Of major importance in this area has
51 been the application of highly sensitive detection techniques including gas chromatography-
52 combustion-isotope ratio mass spectrometry (GC-C-IRMS)[1,2] and more recently liquid
53 chromatography (LC)-IRMS approaches.[6]

54 Initial SIP studies focussed on identification of microorganisms that utilised specific
55 substrates, such as soil dwelling methanotrophic bacteria. Interest in soil methanotrophs
56 stems from the fact that such bacteria occur in every soil order and are an important sink for
57 atmospheric CH_4 in well-aerated soils (high affinity methanotrophs)[7] and a highly efficiency
58 filter that consumes >90% of upward diffusing CH_4 (low affinity methanotrophs) in soils
59 where a sub-surface CH_4 source exists (e.g. landfill cover soils) or *in situ* CH_4 production
60 occurs (e.g. natural wetlands) [8,9,10] Notable successes for SIP in this area include the
61 identification of unculturable high affinity methanotrophic bacteria via PLFA ^{13}C-labelling in
62 well-drained non-agricultural soils.[2] Subsequently, SIP has been extended to quantify
63 methanotroph biomass populations through time series $^{13}CH_4$ PLFA labelling.[11]
64 Interestingly, despite their importance as a carbon sink, there has been little study of soil
65 methanotrophs in ecological contexts as a potential source of soil organic matter. The
66 quantity of carbon cycled via high and low affinity methanotrophy in soils is globally
67 significant,[12,13] yet the fate of that carbon remains largely unknown and unquantified.
68 Understanding the sources and stability of organic carbon in soils is a prerequisite for

69 development of realistic global carbon cycle models that contain fully coupled atmosphere-
70 biosphere-geosphere interactions.

71 Significantly, it has become apparent that long-term time series $^{13}CH_4$ labelling has
72 the potential to yield a wide range of additional information, including: (i) kinetics of ^{13}C
73 uptake, (ii) mechanisms of C incorporation, and (iii) C flow pathways and turnover in soil.
74 For example, in a time series $^{13}CH_4$-incubation study of methanotrophic bacteria in volcanic
75 soils from Tenerife, Spain high concentrations of ^{13}C-label were incorporated into
76 methanotrophic PLFAs.[14] Due to the high levels of ^{13}C- incorporation of ^{13}C, at later stages
77 of the $^{13}CH_4$ incubation, ^{13}C-label was detected in non-methanotrophic fungal biomarkers
78 (e.g. $C_{18:2}$) providing a clear indication of how this approach could be used to investigate
79 pathways of C flow through soil microbial communities.

80 In this study we have combined two previous SIP methods i.e. short pulse-chase
81 experiments and long-term continuous labeling approaches to study biosynthesis and C
82 uptake, to enable a comprehensive study of methanotroph C uptake and CH_4 derived C
83 transport and sequestration through the soil food web. A new long-term ^{13}C-labelling
84 approach has been applied whereby $^{13}CH_4$ was switched to CH_4 containing natural abundance
85 levels of ^{13}C and ^{12}C when full labelling was achieved (as indicated by a maximum in the ^{13}C
86 label incorporation curve; Fig. 1). The incubation was then continued to monitor C turnover
87 of the incorporated ^{13}C-label (hence the term ‘stable isotope switching’ (SIS)). This approach
88 allows short, medium and long-term processes involved in the uptake and turnover of C to be
89 investigated in detail. This continuous labelling method utilises flow-through incubation
90 system and differs from pulse labelling experiments,[6] which typically are conducted over
91 much shorter timescales. The ability to supply a stable flow of isotopically labelled gas at a
92 concentration similar to natural environmental conditions limits disruption to the soil

93 ecosystem and problems associated with selective fertilisation or competitive inhibition of the
94 soil microorganisms.

95 To demonstrate the potential of this technique we conducted a detailed SIS
96 investigation on soils from the Odcombe landfill site (Somerset, UK) which had a previous
97 study[15] has shown to contain a significant population of methanotrophic bacteria. In this
98 methodological paper we document the details of the SIS approach and summarise the range
99 of data produced by SIS to demonstrate its many potential applications. Full datasets, detailed
100 statistical analyses and consideration of all compound classes investigated will be reported in
101 a subsequent communication.

102

103 **Experimental**

104 **Site -** Multiple soil cores (5 cm diameter, 10 cm depth) were collected from two sites at the
105 Odcombe landfill (SW England, 50°56’45”N -2°42’19”W) in March 2007. The landfill
106 consists of a terraced area that was formed by the stacking of waste, which was later capped
107 with sand and clay. The soil cap was sampled from an area of high CH_4 emissions adjacent to
108 a vent previously identified by Crossman *et al.*[15] In this earlier study, conventional PLFA-
109 SIP identified significant populations of low affinity methane oxidising bacteria similar to
110 known Type II methanotrophs, making the cap soil ideally suited for this trial SIS study
111 because of the potential for incorporation of significant amounts of CH_4-derived C into the
112 soil food web. Additionally, landfill cap material is a unique aerobic mineral soil where
113 methanotrophs are a major component of soil microbial biomass, facilitating both rapid and
114 comprehensive labelling of the methanotrophic population and enabling sensitive and
115 selective tracking of the fate of the ^{13}C-signal.

SIS CH_4 incubation

Soils were incubated in a flow through incubation chamber that has been previously described in Maxfield *et al*.[11] All soils were sieved (<2 mm) and incubated in petri dishes (ca. 20 g) over a time course of 154 days. Synthetic air premixed with CH_4 (1.33%) was flowed continuously through the chamber to maintain a steady CH_4 mixing ratio similar to that measured at the landfill site. For the initial 50 days of the incubation 1% of the CH_4 was $^{13}CH_4$ (mixed from > 99% ^{13}C; CK Gas Products Ltd, Hook, UK; equivalent to 133 ppmv $^{13}CH_4$). Following 50 days of incubation the input of $^{13}CH_4$-bearing gas was discontinued and switched to a pre-mixed supply of CH_4 (13300 ppmv) containing natural abundance levels of ^{13}C and ^{12}C. The gas flow rate through the chamber was maintained at 44 mL min^{-1} throughout the entire incubation period, which flushed the entire headspace (63 L) every 24 h. Soil samples were removed in triplicate and from random positions in the incubator at regular intervals during the incubation period at times of 0, 3, 6, 9, 12, 18, 21, 27, 38, 50, 53, 56, 65, 85, 117, 154 days. The initial moisture content of the soil was maintained through the regular addition of double-distilled water (DDW) to each sample (determined gravimetrically). All samples removed from the incubator were stored at -20°C until analysed. Soil samples were freeze-dried and ground prior to extraction and analysis for PLFAs, total amino acids (AAs), total monosaccharides glycolipids (GLFAs), free fatty acids (FFAs), hopanoids, sterols, *n*-alkanes and *n*-alkanols.

Lipid extraction and fractionation

All soils were extracted using a modified Bligh Dyer extraction methodology as described previously.[11] Lipid fractionation was obtained using a modified silicic acid fractionation protocol[16] to yield three fractions: simple lipids, glycolipids and phospholipids. Simple

140 lipids were further fractioned using silicic acid columns into four fractions; hydrocarbons
141 (eluted with hexane, 4 mL), ketones and wax esters (dichloromethane (DCM), 6 mL),
142 alcohols (DCM/methanol (MeOH), 1:1 *v/v*, 4 mL), and polar poly-functionalised compounds
143 (MeOH, 4 mL). Alcohols and hydrocarbons were further separated by urea adduction to
144 separate cyclic from acyclic components.[17]

145

146 **Lipid derivitization**

147 The PLFA, GLFA and FFA fractions were methylated with BF_3/MeOH (14% *w/v*) by heating
148 at 70°C for 1 h. Fatty acid methyl esters (FAMES) were dissolved in *n*-hexane for analysis by
149 GC, GC/mass spectrometry (MS) and GC/C/IRMS. All neutral polar lipids were derivatized
150 with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) prior to analysis by GC, GC/MS and
151 GC/C/IRMS.

152

153 **Extraction and derivatization of carbohydrates**

154 The method of Blakeney *et al.*[18] modified by Docherty *et al.*[19] was employed to prepare
155 alditol acetate derivatives of total monosaccharides. Soils were hydrolysed with H_2SO_4 and
156 the released monosaccharides reduced with $NaBH_4$. Excess $NaBH_4$ was destroyed by the
157 addition of glacial acetic acid to reduce the liberated monosaccharides to their corresponding
158 alditols. The alditols were acetylated by reaction with *N*-methylimidazole and acetic
159 anhydride. A standard mix of rhamnose, fucose, galactose, mannose, xylose, arabinose
160 glucose, inositol and pentaerythritol was prepared according to Docherty *et al.*[19] The DDW
161 (400 μl) was added to the dry monosaccharide standards which were derivatized as above. A

162 20 μg μl^{-1} internal standard (I.S.) was prepared by dissolving 0.04 g pentaerythritol in 2 ml 1
163 M NH_3 solution.

164

165 **Extraction and derivatization of amino acids**

166 An internal standard of nor-leucine (0.2 mg ml^{-1}) was prepared for quantification of AAs.
167 Each AA produced a different GC-FID response which required correction by the application
168 of response factor. The FID response factors were determined from a standard solution of
169 AAs of known concentration according to the method of Corr *et al.*[20] Extracted soil samples
170 were hydrolysed with HCl and purified by ion exchange chromatography[21] using Dowex
171 50WX8-400 Ion Exchange Resin (Acros Organics, Geel, Belgium). The purified amino acids
172 were derivatized to N-acetyl methyl esters using acetone, triethylamine and acetic anhydride
173 (5:2:1, *v/v*)[20].

174

175 **Instrumental analyses**

176 **GC** analysis were performed using a Hewlett-Packard Series 5890 Series II gas
177 chromatograph (Agilent Technologies UK Ltd., Edinburgh, UK) equipped with a flame
178 ionisation detectors (FID) using H_2 carrier gas (10 psi). Non-polar fractions were analysed
179 using a Chrompack CPSil-5CB (50 m x 0.32 mm i.d. x 0.12 μm film thickness). The
180 temperature conditions were 50ºC to 200ºC at 10º C min^{-1}, to 300ºC at 3ºC min^{-1} (held for 20
181 min). Polar compounds were analysed using a Varian VF23ms (Varian BV, Middelburg, The
182 Netherlands) 50% cyanopropyl equivalent fused-silica column (60 m x 0.32 mm i.d. x 0.25
183 μm film thickness). The temperature conditions for fatty acid derivatives were 50ºC (2 min)
184 to 100ºC at 15ºC min^{-1}, to 240ºC at 4ºC min^{-1} (held for 20 min). The temperature programme

185 for monosaccharides was 50ºC (1 min) to 200ºC at 20ºC min^{-1}, to 230ºC at 4ºC min^{-1}, (held
186 for 22 min). The temperature programme for amino acids was 40ºC (1 min) to 120ºC at 15ºC
187 min^{-1}, to 190ºC at 3ºC min^{-1}, to 250ºC at 5ºC min^{-1} (held for 20 min).

188 **GC-MS** analyses were performed using a Thermo Finnigan Trace GC-MS (Thermo Fisher
189 Scientific, Hemel Hempstead, UK). All the GC conditions were the same with the exception
190 of helium being used as carrier gas. The interface was held at the maximum oven
191 temperature, the ion source was held at 200°C and the quadropole mass analyser operated in
192 EI mode scanning over the range *m/z* 50-650 at 1.7 scans s^{-1}. The emission current was
193 maintained at 300 μA and electron energy was 70 eV. The data were acquired and analysed
194 using the Excalibur software Version 1.2 (Thermo Fisher Scientific, Hemel Hempstead, UK).

195 **GC-C-IRMS** analysis were carried out using a Varian 3500 GC (Varian BV, Middelburg,
196 The Netherlands) coupled to a Finnigan MAT DELTA-S isotope ratio mass spectrometer
197 (Thermo Fisher Scientific, Hemel Hempstead, UK). Analytes in He were combusted to CO_2
198 using a modified Type I Finnigan MAT combustion interface with a CuO/Pt combustion
199 reactor set at a temperature of 850°C prior to entry into the mass spectrometer source via an
200 open split. The ionisation source electron energy was 100 eV with an electron current of 1
201 mA. Detection was via 3 Faraday cup collectors set at *m/z* 44, 45 and 46. The
202 chromatographic conditions were the same as those described previously for GC analyses.
203 All samples were analysed in duplicate to verify reliability of $\delta^{13}C$ values. Samples were
204 calibrated against reference CO_2 of known isotopic composition which was introduced
205 directly into the source three times at the beginning and end of every run. Compound specific
206 IRMS performance was determined using a suite of externally calibrated reference fatty acid
207 methyl esters. Analytical precision was <0.5 ‰ (± 1 standard deviation) based upon replicate
208 analysis of reference standards (n=5).

209 **EA-IRMS** analysis of freeze-dried and ground soil samples was conducted using a
210 Eurovector C-N (Eurovector, Milan, Italy) elemental analyser interfaced to an Isoprime
211 (Isoprime Ltd., Manchester, UK) stable isotope ratio mass spectrometer (IRMS). Samples
212 were weighed (1–2 mg), placed into tin capsules and combusted. $\delta^{13}C$ values of the resultant
213 CO_2 from combustion were determined at the NERC Stable Isotope Facility at CEH
214 Lancaster with an analytical precision of <0.15 ‰ (± 1 standard deviation, n=16).

215

216 **Statistical analyses**

217 Two approaches were used to analyse the ^{13}C-label incorporation curves. Linear and non-
218 linear regressions were performed using GraphPad Prism version 5.02 for Windows
219 (GraphPad Software, San Diego, CA, USA), R 2.8.1 (R foundation), and Microsoft Excel
220 (Microsoft, Redmond, WA, USA). Least squares linear regression analysis was applied to %-
221 incorporation data from the first 9 days of the incubation to obtain initial zero-order rate
222 constants, whereas separate non-linear regressions were applied to incorporation data from
223 the first 50 d of the incubation before the switchover to unlabelled CH_4, and the C turnover
224 data from 50 d to 150 d. The equations of the fitted lines were of the form:

225 Incorporation: $$F = F^0 + (P - Y^0) * (1 - \exp(-K * t)) \quad (1)$$

226 Decay: $$F = (F^0 - P) * \exp(-K * t) + P \quad (2)$$

227 Where F is the fraction of excess ^{13}C incorporated into organic material, t is the incubation
228 time in days, R^0 is the initial R value at initial t, P is the plateau at maximum ^{13}C
229 incorporation (incorporation) or maximum ^{13}C loss (decay) and K is the first order rate
230 constant expressed in inverse days.

231

232 **Results and Discussion**

233 The SIS approach differs from pulse-chase methods in two key respects: (i) substrate delivery
234 is maintained throughout the experiment at a constant concentration in order to establish
235 equilibrium conditions between substrate and the primary substrate consumer population thus
236 allowing evaluation of substrate cycling purely based on changes in isotopic labelling
237 patterns in different endogenous chemical species, and (ii) the only change in substrate
238 delivery is in its stable isotopic composition, i.e. ^{13}C-enriched to natural abundance, which is
239 switched when isotopic equilibrium with the primary consumers has been established. As a
240 result it is possible to study both assimilation and decay of the ^{13}C-label in a wide range of C
241 pools and molecular species (including biomarker compounds) within a complex ecosystem,
242 in order to provide insights into assimilation pathways, kinetics of turnover and quantitative
243 estimates of pool sizes.

244

245 **Methanotrophic bacteria as primary consumers**

246 Soil PLFA profiles provide general information about soil microbial community structure,
247 diversity and size. PLFA nomenclature indicates the length of the C chain (first number), the
248 number of double bonds (number after the colon), the position of the double bond
249 (ω, counted from the defunctionalised end of the molecule) and geometry (for full details see
250 Zelles).[22] The extremely high abundance of 18:1ω7c observed in the Odcombe vent PLFA
251 profile (Fig. 2) indicates the likely dominance of α-proteobacterial (type II) methanotrophs
252 linked to the high CH_4 flux at the site. This predominance was confirmed by GC-C-IRMS
253 analysis of the PLFA fraction following the $^{13}CH_4$-enriched incubation which also showed
254 with the largest proportion of ^{13}C was incorporated into PLFA 18:1ω7c. The prevalence of
255 methanotrophic bacteria resulted in highly ^{13}C-labelled soils following the $^{13}CH_4/^{12}CH_4$

256 incubation enabling methanotroph C derived from $^{13}CH_4$ to be traced through the complex
257 soil food web.

258

259 **Stable Isotope Switching**

260 The extracts from the ^{13}C-labelled soils were analysed by GC-C-IRMS to determine $\delta^{13}C$
261 values to quantify and monitor the fate of metabolised CH_4 across a wide range of compound
262 classes representative of a range of soil biota. Whilst PLFAs are the most commonly studied
263 biomarkers in methanotrophic bacteria SIP studies we and others have shown previously the
264 potential for linking hopanoids with methanotrophic bacteria through $^{13}CH_4$-labelling
265 studies.[23,24,25] However, the purpose of SIS is to move beyond functional taxonomic
266 profiling to explore more fully C cycling and soil microbiological function. Thus, we have
267 conducted a comprehensive survey of methanotroph-derived biochemicals tracing ^{13}C-label
268 into amino acids, carbohydrates, glycolipids, free fatty acids, alcohols, alkanes, hopanoids,
269 sterols and resorcinols (Fig. 3). Different compound classes represent soil C pools of
270 differing stability and recalcitrance. Further, the magnitude of methanotroph C turnover by
271 the soil microbial community suggests that those biochemicals which display little or no ^{13}C-
272 labelling are not closely linked with soil C turnover by soil microorganisms, and thus
273 represent either stable soil C pools receiving little fresh C input, or are soil C pools with a
274 conserved source of C. In addition the total incorporation of ^{13}C into the bulk soil also was
275 assessed. Comparison with this bulk $\delta^{13}C$ value indicates the recalcitrance of individual C
276 pools relative to the total pool of soil organic C (Fig. 3).

277 Figure 4 shows an overview of ^{13}C-label assimilation and dissimilation profiles for a
278 range of selected soil compounds from several of the main compound classes: 16:1ω7c
279 PLFA, C_{31} homohopenol, glucose, valine and the bulk soil. Whilst there are clear differences

280 in the extent of ^{13}C-labelling of these compound classes the δ^{13}C curves exhibit the general
281 shape predicted from the experimental design (Fig. 1) with C turnover following a first order
282 rate dissociation curve (Fig. 4, right hand side). However, it is important to note both the
283 differences in the relative uptake of ^{13}C into the different soil compound classes and the wide
284 range of turnover rates which reflects the refractiveness of different biochemicals analysed in
285 this study.

286

287 **Soil Food Web**

288 As with previous conventional time series labelling studies ^{13}C-label uptake monitored by
289 GC-C-IRMS and expressed by δ^{13}C values represents the magnitude of ^{13}C-label uptake into
290 a specific compound as a proportion of the total concentration of that compound. Thus, δ^{13}C
291 values do not indicate the absolute amount of ^{13}C-label present in a specific pool but rather
292 the proportion of that C pool that is derived from the ^{13}C-labelled C source. Hence SIS
293 provides a potential new tool for use in soil food web studies which can be employed to
294 determine the rate of C flow through a soil microbial network and the rate of *in situ*
295 biosynthesis of specific compounds within that network. Figure 4 indicates that a significant
296 proportion of PLFAs within the Odcombe soil were synthesised by methanotrophs directly
297 from CH_4. All PLFAs displayed uptake of the ^{13}C-label indicating both the high magnitude of
298 initial $^{13}CH_4$ incorporation and the extensive redistribution of assimilated C within the soil
299 system. The rates and magnitudes of ^{13}C uptake vary widely indicating that the PLFA
300 producing organisms differed in terms of their usage of CH_4 derived C in biosynthesis, and
301 the biochemical proximity of different PLFAs to the ^{13}C source ($^{13}CH_4$). To more easily
302 visualize PLFA ^{13}C incorporation profiles those data are shown separately (in Fig. 5)
303 highlighting the differences in ^{13}C uptake rate between different groups of PLFA sources.

304 Despite their lack of taxonomic specificity PLFAs have been widely used to broadly
305 characterize microbial populations[22] and as such PLFAs are an extremely effective tool for
306 identifying active bacterial groups utilizing ^{13}C-labelled tracer compounds. For example,
307 PLFAs commonly linked to methanotrophic bacteria (18:1ω7c, 16:1ω7, 16:1ω5) most rapidly
308 incorporate the ^{13}C-label (Fig 5, panel a). The primary methanotroph PLFA in this soil,
309 18:1ω7c also reaches a plateau before the $^{13}CH_4$ supply was discontinued following 50 days
310 incubation, indicating maximum ^{13}C-labeling of the methanotroph population and
311 establishment of isotopic equilibrium within the primary consumer population. Primary
312 methanotrophic PLFAs can be separated from PLFAs that incorporated a lower proportion of
313 ^{13}C at a slower rate, the latter likely being indicative of secondary (Fig 5, panel b) and tertiary
314 consumers (Fig 5, panel c). Secondary and tertiary consumers incorporate ^{13}C-label at much
315 reduced rates compared to primary consumers. For example following 18 days incubation
316 under $^{13}CH_4$ primary consumer $\delta^{13}C$ values increased by +100 to +300 ‰ (Fig. 5a),
317 secondary consumer $\delta^{13}C$ values increased by +30 to +60 ‰ (Fig. 5b), and tertiary consumer
318 $\delta^{13}C$ values increased by +10 to +20 ‰ (Fig. 5c). Because microorganisms linked with non-
319 primary methanotroph PLFAs are not obtaining ^{13}C label direct from $^{13}CH_4$, the likely
320 alternative ^{13}C-label redistribution mechanisms are metabolite release and uptake, necromass
321 grazing and direct predation.

322 Furthermore, it was observed following prolonged incubation under $^{13}CH_4$ that a
323 significant proportion of PLFA C was derived from CH_4. Accounting for the fact that the ^{13}C-
324 enriched CH_4 used in this study was only 1% enriched in $^{13}CH_4$, and extrapolating the results
325 to take this dilution into account, following 38 days of the incubation 45% of the C present in
326 the Odcombe soil PLFAs was derived from CH_4. This proportion includes all PLFAs
327 extracted from the soil, which suggests that >45% of total bacterial PLFA C consists of C
328 derived from CH_4. Even allowing for the high abundance of methanotrophs in the Odcombe

329 soil this is an unexpectedly high proportion of bacterial C after a relatively short incubation
330 periods, suggesting that microbial communities in soil and sedimentary environments where a
331 significant source of CH_4 is present must utilise CH_4 as a major source of C and energy for
332 growth. This high proportion of C is consistent with the appreciable amount of CH_4-derived
333 C that is incorporated into certain lake food webs where CH_4 production is prevalent in
334 anoxic sediments.[26] Whilst there is a wide range of estimates between different lakes,
335 chironomid larvae have been observed as a primary conduit for the trophic transfer of
336 biogenic CH_4 gaining >60% of their C from CH_4[27] and zooplankton in small boreal lakes
337 ~50% through grazing on methanotrophs.[28] In these lake studies trophic C transfer was
338 determined via physical separation of the organisms of interest prior to bulk isotopic
339 measurement by EA-IRMS. The work reported herein is the first assessment of the proportion
340 of CH_4 derived C that is transmitted through microbial food webs, which has been made
341 possible through the application of SIS in combination with GC-C-IRMS analysis of a wide
342 range of soil biochemical components.

343

344 **C sequestration**

345 In addition to C uptake kinetics, the SIS method also enables the study of rates of C turnover,
346 redistribution and sequestration. Figure 4 (right hand side) shows C turnover of PLFAs
347 following the change from incubation under $^{13}CH_4$ to natural abundance CH_4 and the fitted
348 first order exponential dissociation curves used to determine C turnover rate constants. Box
349 plots of C turnover for each group of compounds analyzed by GC-C-IRMS indicate no
350 observable relationship between C assimilation and turnover (Fig. 3). There is a high degree
351 of variability in both C turnover rates and loss both within and between various compound
352 classes. Although CH_4 is a significant source of C in the Odcombe soils, a large amount of

353 the C utilized in cellular biosynthesis is rapidly lost from the system and not sequestered in
354 long-term C pools. The C pools where C retention is poor include PLFAs, GLFAs, FFAs,
355 carbohydrates, resorcinols and *n*-alkanols, which all lose >60% of their CH_4-derived C after
356 100 days of incubation under natural abundance CH_4. These losses suggest that despite
357 significant initial incorporation of CH_4-derived C, little of the C will be retained long-term
358 within the Odcombe landfill cover soils. The most recalcitrant forms of C include protein-
359 derived amino acids, steroids and hopanoids (Fig. 3). Notably, a wide range of turnover rates
360 were observed for the hopanoids, and the diversity is likely due to functionalised hopanoids
361 being converted to their de-functionalised more stable analogues, indicating long residence
362 times for the pentacylcic hopanoid core structures.

363 First order dissociation curves were fitted to ^{13}C decay curves (Fig. 4) and associated
364 rate constants were used to calculate CH_4-derived C residence times in the Odcombe soils.
365 Total bulk soil ^{13}C had a half-life of 68 days and 47% of the total C derived from CH_4 was
366 retained within the soil after 100 days. Extrapolation of these values indicates that 1 year after
367 SIS just 19% of the CH_4-derived C will remain in the Odcombe soil, with 81% being released
368 primarily as CO_2 via aerobic soil respiration.

369

370 **Conclusions**

371 This study demonstrates that SIS has considerable potential as a new method for determining
372 the kinetics of soil C uptake, turnover, release and sequestration at the molecular level in
373 complex soil and sedimentary matrices. Our findings indicate that in the Odcombe landfill
374 cover soil 45% of bacterial membrane lipid C was directly derived from CH_4 and at the end
375 of the experiment 47% of bulk soil C derived from CH_4 was retained within the soil. In this
376 communication we have demonstrated the potential power of this technique to resolve

377 discreet C cycling processes within a complex environmental sample. Whilst we have
378 initially focussed on the soil food web in a landfill cover soil, SIS has considerable potential
379 for identifying and elucidating hitherto elusive aspects of nutrient cycling in many different
380 environments using a range of gaseous or liquid substrates, and will lead to new molecular
381 level estimates of ecosystem nutrient dynamics for use in process-based models of element
382 cycling. Previous applications of SIP have utilised a range of ^{13}C-labelled substrates
383 including acetate, glucose, methanol, CO_2 (both directly and indirectly via individual plants;
384 for full overview see Maxfield and Evershed and references therein).[29] The main drawback
385 of the approach is the requirement to incubate soils removed from their natural habitat for
386 long periods, which has the potential to cause changes in the composition of the wider soil
387 microbial community. This issue could be overcome by conducting SIS incubations *in situ*
388 (i.e., in field setting, using an appropriately designed isotope delivery system).[30] The SIS
389 technique could be readily applied to other light rare isotopes including ^{2}H and ^{15}N and we
390 recently developed an aqueous-based re-circulating isotope delivery system that can be
391 employed to conduct SIS experiments using a wide range of different substrates.

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495 **Figure Captions**

496 **Figure 1:** Schematic representation of stable isotope switching (SIS) experiment based upon
497 previously observed (light grey) and theoretical (dark grey) curves indicating how ^{13}C-label
498 from $^{13}CH_4$ is incorporated by methanotrophs and turned-over within soil ecosystems. The
499 timescale to achieve full labelling of the target population will vary depending upon the
500 isotopically labelled substrate composition, concentration, delivery method and the nature of
501 the environmental sample to be studied.

502 **Figure 2:** Partial gas chromatogram of the Odcombe landfill soil PLFA fraction (T_0 d).
503 Where 1, C_{19} alkane; 2, *i*14:0; 3, *a*14:0; 4, *i*15:0; 5, *a*15:0; 6, 15:0; 7, *i*16:0; 8, 16:0; 9,
504 16:1ω7, 16:1ω5; 10, *i*17:0; 11, *a*17:0; 12, 17:0; 13, 17:1ω8; 14, 18:0; 15, 18:1ω7; 16, 18:2;
505 17, 20:0; 18, 18:3; 19, 11-CH_3O-17:0; 20, 22:0; 21, br23:0; 22, 23:0; 23, 24:0. See text for
506 explanation of PLFA nomenclature.[26]

507 **Figure 3:** Top panel - ^{13}C-label uptake rate (zero order) from $^{13}CH_4$ into a wide range of soil
508 biochemicals; Bottom panel - ^{13}C-label turnover (first order) following SIS to natural
509 abundance. Whiskers represent the lowest data point still within 1.5 of the interquartile
510 range of the lower quartile, and the highest data point still within 1.5 of the interquartile range
511 of the upper quartile.

512 **Figure 4:** Mean $\delta^{13}C$ values for bulk C and selected components extracted from Odcombe
513 landfill soil following incubation under 1.3% CH_4. Error bars represent ±1 standard deviation
514 (n = 3). Left hand side = 1% enriched in $^{13}CH_4$. Right hand side = CH_4 with a natural
515 abundance of ^{13}C and ^{12}C. C decay curves were fit to a first order dissociation rate constant
516 (Equation 2)

517 **Figure 5:** ^{13}C-label incorporation by selected PLFAs. (a) primary consumers, (b) secondary
518 consumers, and (c) tertiary consumers. Note the different y-axis scales for plots a, b and c.
519 Error bars represent ±1 standard deviation from the mean (n = 3).

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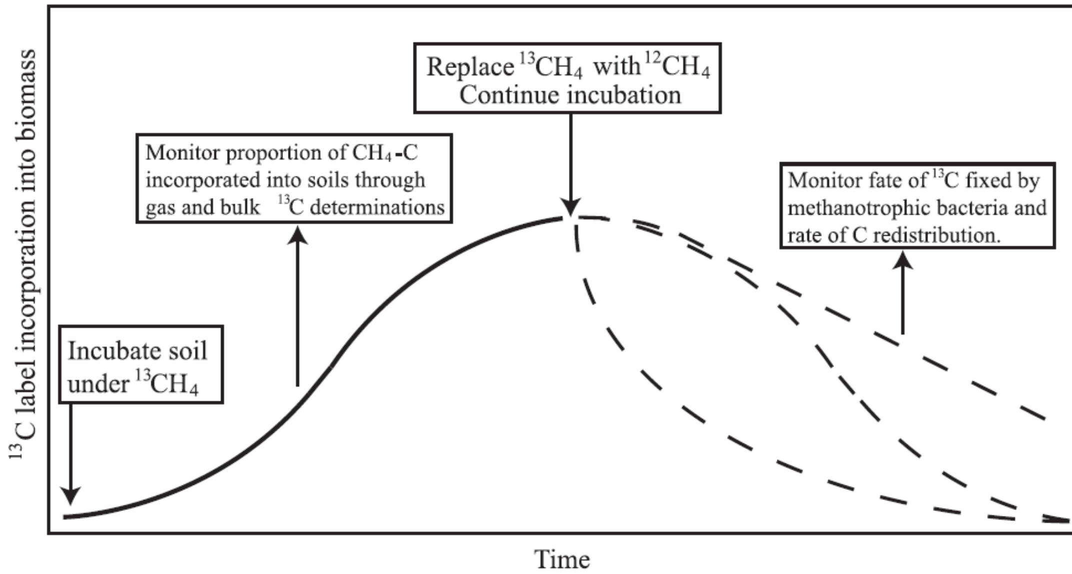
Methanotrophic biomass (1° uptake)

Carbon flow to non-methanotrophic pools (2°, 3° uptake)



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537 Figure 1: Maxfield *et al.*

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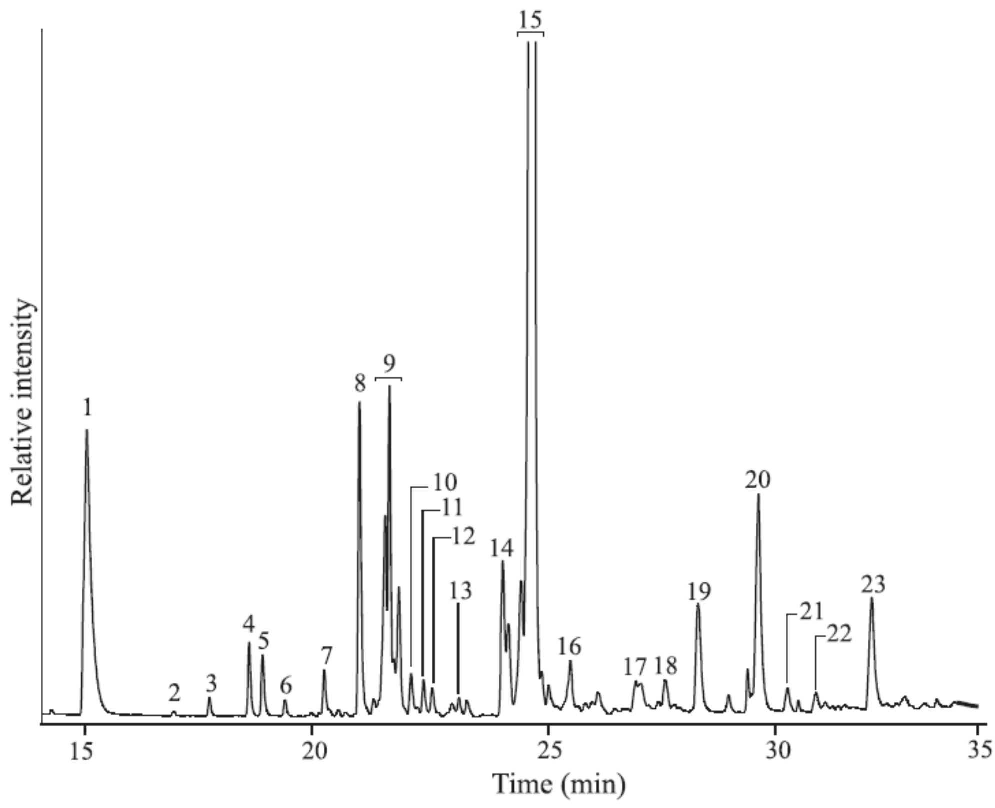

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547 Figure 2: Maxfield *et al.*

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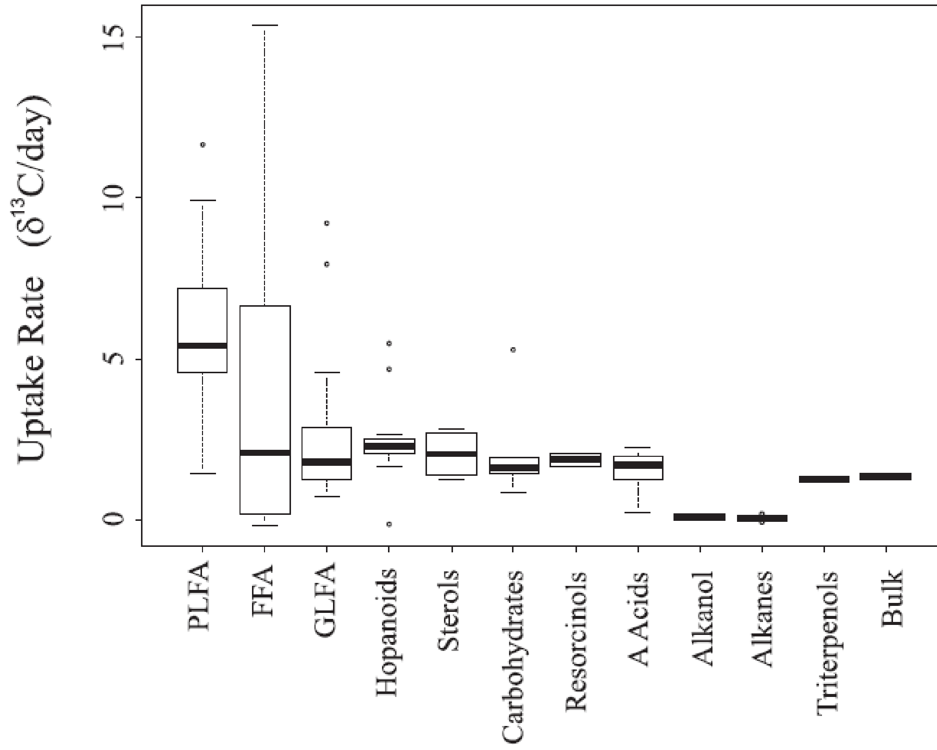

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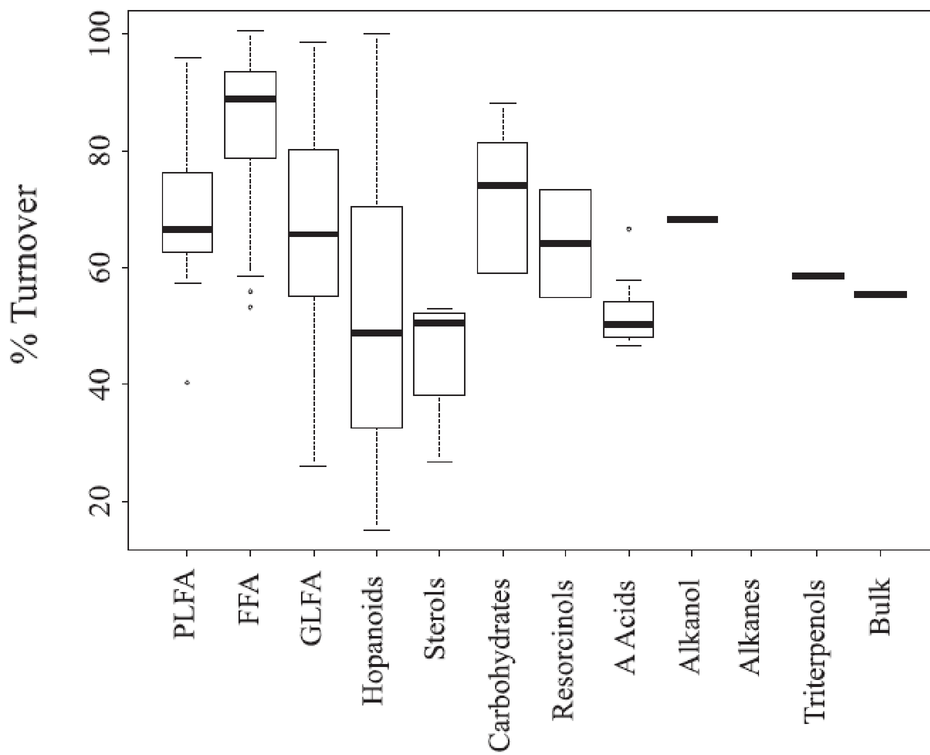

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557 Figure 3: Maxfield *et al.*

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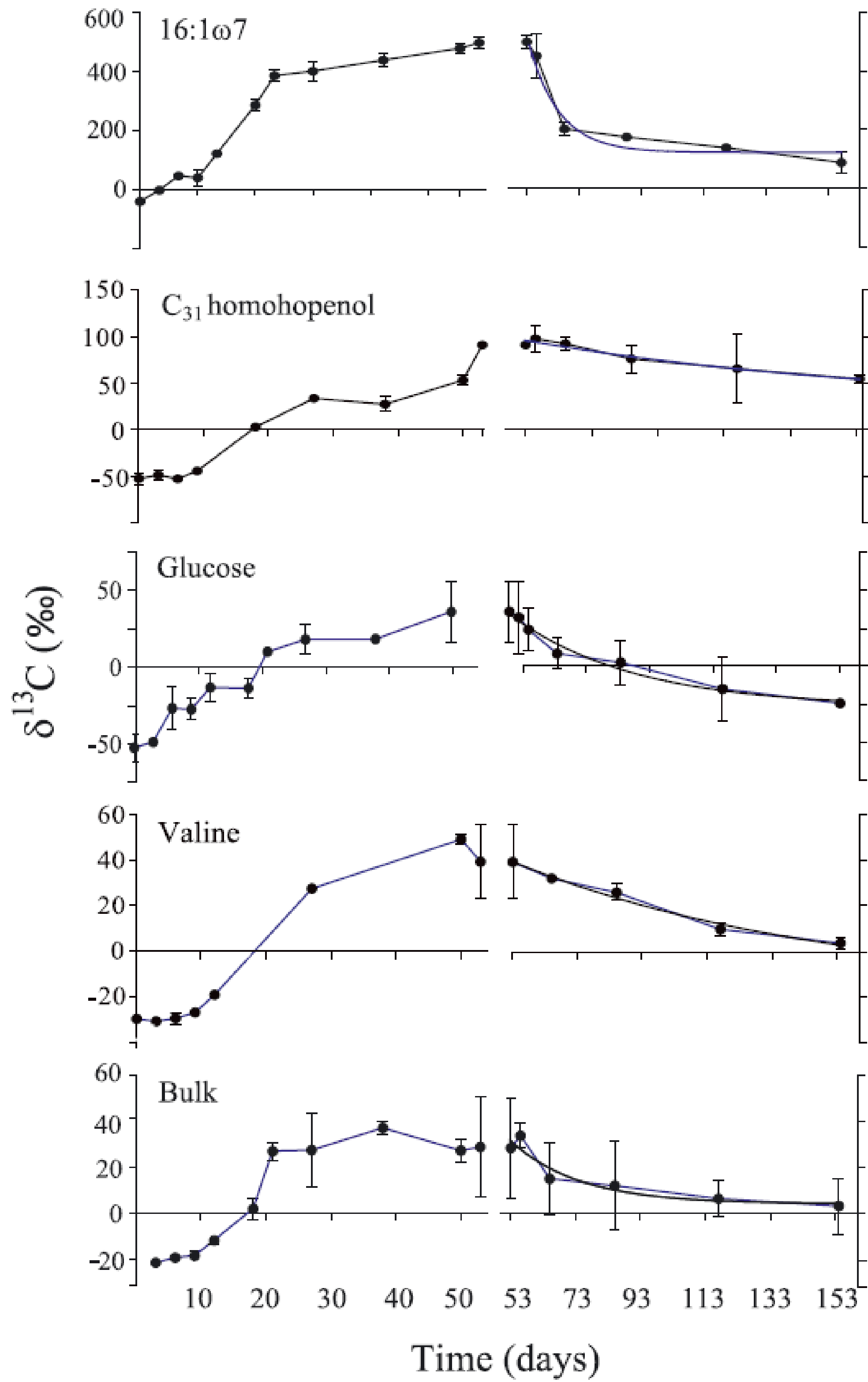

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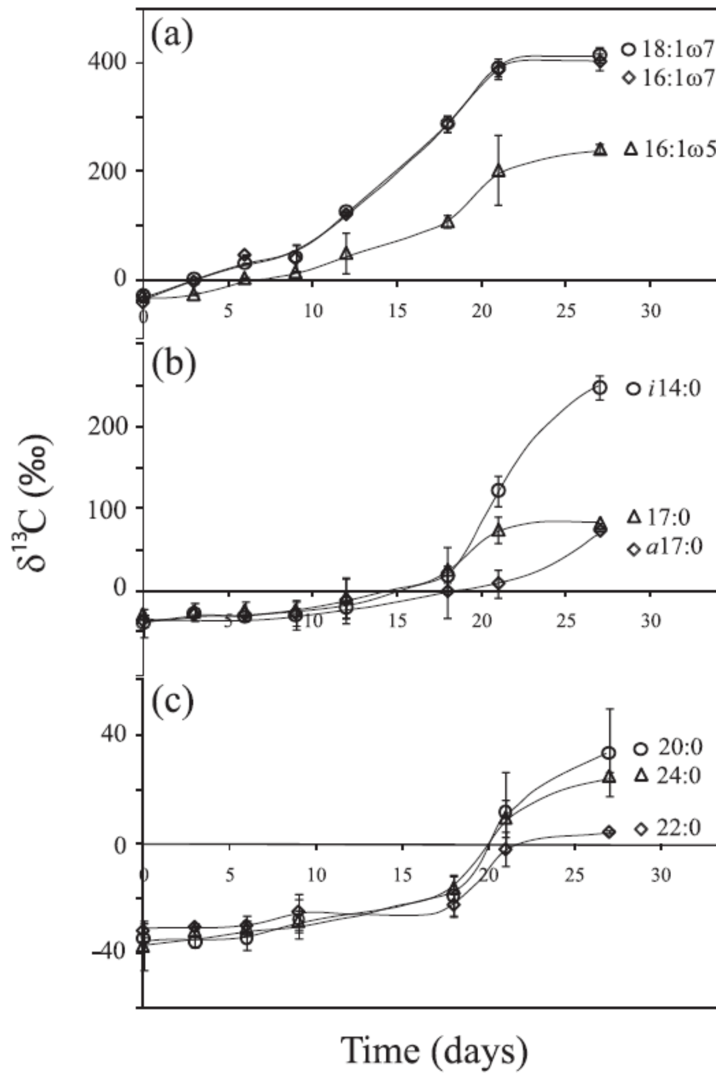
561 Figure 4: Maxfield *et al.*



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563 Figure 5: Maxfield *et al.*