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# Apportioning bacterial carbon source utilization in soil using $^{14}C$ isotope analysis of FISH-targeted bacterial populations sorted by Fluorescence Activated Cell Sorting (FACS): $^{14}C$-FISH-FACS

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**Running title:** Apportioning bacterial carbon source utilization in soil

**Originality-significance statement:** We present a novel combination of methodology ($^{14}C$-FISH-FACS) to address an unresolved need in microbial ecology: the ability to quantify *in situ* microbial substrate carbon use at the population level within a whole system carbon mass balance. Using $^{14}C$-FISH-FACS, we demonstrate assimilation of salicylic acid by targeted *Pseudomonas* spp. and *Burkholderia* spp. populations. In conjunction with analysis of the

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taxonomic distribution of the salicylic acid biodegradation trait, we evidence that the ability of the targeted populations to capture a large proportion (~50%) of the added C was not due to conservation of this trait within the targeted group but instead due to competitiveness of this population for the added carbon. The sensitivity of $^{14}$C-FISH-FACS and its compatibility with obtaining a full-system mass balance allows a quantitative dissection of C flow within the microbial biomass black box that has hitherto not been achieved to the same detail. $^{14}$C-FISH-FACS will enable advances in understanding of population-specific C competitiveness, C use traits and their role in controlling overall C dynamics and microbial community composition in soil and rhizosphere.



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**Summary**

An unresolved need in microbial ecology is methodology to enable quantitative analysis of *in situ* microbial substrate carbon use at the population level. Here, we evaluated if a novel combination of radiocarbon- labelled substrate tracing, Fluorescence *in situ* Hybridisation (FISH) and Fluorescence-Activated Cell Sorting (FACS) to sort the FISH-targeted population for quantification of incorporated radioactivity ($^{14}$C-FISH-FACS) can address this need. Our test scenario used FISH probe PSE1284 targeting *Pseudomonas* spp. (and some *Burkholderia* spp.) and salicylic acid added to rhizosphere soil. We examined salicylic acid-$^{14}$C fate (mineralized, cell-incorporated, extractable and non-extractable) and mass balance (0-24 h) and show that the PSE1284 population captured ~50% of the Nycodenz extracted biomass $^{14}$C. Analysis of the taxonomic distribution of the salicylic acid biodegradation trait suggested that PSE1284 population success was not due to conservation of this trait but due to competitiveness for the added carbon. Adding 50KBq of $^{14}$C sample$^{-1}$ enabled detection of $^{14}$C in the sorted population at ~60-600 times background; a sensitivity which demonstrates potential extension to analysis of rarer/ less active populations. Given its sensitivity and compatibility with obtaining a C mass balance, $^{14}$C-FISH-FACS allows quantitative dissection of C flow within the microbial biomass that has hitherto not been achieved.

**Introduction**

Soil microorganisms play a critical role in driving the cycling of soil organic carbon (Nielsen et al., 2011; Gougoulias et al., 2014; Wieder et al., 2015) and in recognition of this importance there has been considerable discussion about the representation of microbial-scale processes and



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the composition of the microbial biomass in carbon cycle models (McGuire and Treseder, 2010; Trivedi et al., 2013; Wieder et al., 2015). Such representation of microbial communities requires knowledge of the relationship between microbial community composition and the rate of carbon cycling processes (Trivedi et al., 2013). In a synthesis of findings from studies that have investigated the relationship between soil biodiversity and ecosystem functions related to C cycling, Nielsen et al. (2011) have concluded that whilst the overall richness of soil communities may have minimal effects on soil C cycling, changes in community composition that result in a changed abundance and activity of particularly influential species may be much more important. Therefore, it might be species traits rather than species richness per se that primarily control C cycling processes (Nielsen et al., 2011). For example, the efficiency with which microorganisms convert available C to biosynthesized products versus respired $CO_2$ (i.e. carbon use efficiency) is a trait with the potential to vary at the microbial population level (Geyer et al., 2016) that ultimately influences the proportion of substrate C that is incorporated in to stable soil organic matter (Miltner et al., 2012; Geyer et al., 2016). However, how the use efficiency of carbon and its destination with in the metabolome (i.e. biomass versus exudate production) at the microbial population level scales to influence community and ecosystem level C cycling is underexplored, partly due to technical difficulties in empirically examining the *quantitative* role of individual microbial species in processing C *in situ* (Geyer et al., 2016).

The population specific microbial use of C in the rhizosphere has particular significance for global biogeochemical cycles given the magnitude of the rhizodeposition C flux, its role in priming the decomposition of existing soil organic matter and in driving heterotrophic pathways responsible for the cycling and dissimilation of other elements. We know that through



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rhizodeposition, plants shape their microbiome with dynamic C availabilities selecting for fast-growing, competitive genotypes (Lundberg et al., 2012; Peiffer et al., 2013; Chen et al., 2016). This selection has functional consequences additional to those related to nutrient cycling in terms of the recruitment of microorganisms that are beneficial or deleterious to plant health (Berendsen et al., 2012). Whilst there have been many studies that have examined the impact of plant genotype or plant growth stage on the rhizosphere microbiome, we know less about the competitiveness of specific microbial populations for components of rhizosphere C flow. Such understanding might be indispensable for engineering the rhizosphere for benefit but, again, is partly hampered by technical difficulties in empirically examining the quantitative role of individual microbial species in processing C *in situ*.

A central goal in microbial ecology has long been to link carbon use to specific microbial taxa in the environment and a suite of methods involving the tracing of $^{13}C$- or $^{14}C$ -labelled substrates (reviewed by Gutierrez-Zamora and Manefield, 2010; Musat et al., 2012; Wang et al., 2016) have been developed in order achieve this goal without the need for cultivation and characterization of isolates. These methods have been classified into two groups (Gutierrez-Zamora and Manefield, 2010): (i) isotope probing methods (e.g. DNA-, RNA-, PLFA- and protein-stable isotope probing (SIP)) which allow the identification of functional members of a community without prior knowledge of their identity; and, (ii) probe-based methods with either *in situ* (e.g. microautoradiography–fluorescence *in situ* hybridisation (MAR-FISH), FISH-Raman microspectroscopy, Nano secondary ion mass spectrometry *in situ* hybridization (NanoSIMS-ISH)) or *ex situ* (e.g. CHIP-SIP) hybridisation which require prior knowledge of the identity of the population of focus. The development of these methods has revolutionised microbial



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ecology over the last two decades, however, the methods vary in the extent to which they can *quantitatively* apportion the utilisation of C to specific microbial populations *in situ*. With nucleic acid- based isotope probing methods, it is not generally possible to determine the extent of isotopic incorporation per phylogenetic group due to reliance on PCR for analysis of microbial identity which dilutes the isotopic content of the initial DNA or RNA (Gutierrez-Zamora and Manefield, 2010). In contrast, quantification of isotopic incorporation is possible with Protein-SIP and PLFA-SIP (Gutierrez-Zamora and Manefield, 2010; Lunsmann et al., 2016). Probe-based methods also generally allow, with appropriate calibration, the microbial incorporation of C from C isotope-labelled substrates to be quantified. In "Chip-SIP" isotope array analysis, ex situ hybridisation to a high-density microarray is used to phylogenetically sort extracted rRNA into individual rRNAs, and then their isotopic enrichment is quantified with NanoSIMS (Mayali et al., 2012). In *in situ* methods, isotope incorporation at the single cell level can be quantified by MAR, NanoSIMS or Raman microspectroscopy giving indispensable insight in to microbial processes at the scale approaching that at which they occur (Gutierrez-Zamora and Manefield, 2010; Eichorst et al., 2015).

The above quantitative identity-function methods add a great deal to the methodological tool box in microbial ecology, however, they also have certain limitations. Compared to nucleic-acid based methods, protein-SIP and PLFA-SIP are limited in phylogenetic resolution; labelled peptides or labelled PLFAs can often only be assigned to bacterial families or orders (Jehmlich et al., 2016) or are biomarkers for broad groups of microorganisms (Gutierrez-Zamora and Manefield, 2010), respectively. For probe-based methods, even including Chip-SIP where multiple probes are deployed in parallel, a concern is that taxa coverage is dependent on



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selection of probes and therefore analysis might miss activity in unexpected taxa (Mayali et al., 2012). In addition, the application of NanoSIMS to soil samples is not straightforward due to the large background of soil particles and sensitivity of the technique to sample topographical unevenness and label dilution due to redeposition of sputtered material (Eichorst et al., 2015). Similarly, Raman microspectroscopy also requires cells that are dispersed from soil constituents to retrieve reliable cell spectra (Eichorst et al., 2015). NanoSIMS-ISH, FISH-Raman, Protein-, PLFA- and Chip-SIP also rely on access to costly and specialised analytical instrumentation (LC-MS/MS; GC-c-IRMS; NanoSIMS; Fluorescence-equipped Raman Microscope; Array Synthesis) meaning that access might be an issue for many researchers.

When the interest is quantifying flows of total substrate carbon, however, a large constraint is that methods which quantify C incorporation into specific classes of biomolecules (protein-, PLFA- and Chip-SIP) do not provide a whole-cell view of C incorporation by a microbial group of interest. Extrapolation of C incorporation from the biomolecule to the whole cell level that is prerequisite to apportioning C use to a population within the context of a system mass balance is problematic because, at the cell level, microorganisms will not allocate substrate derived carbon equally within their metabolome and for biomolecule synthesis. Out of the methods reviewed, only the *in situ* methods detecting C isotopic incorporation at the whole cell level (by MAR, NanoSIMS) negate the need to extrapolate from biomolecules to whole cells if trying to understand and quantify the flows of total substrate carbon. However, these methods are limited in the number of cells per sample that can be analysed (Clode et al., 2009; Wang et al., 2016). Consequently, it might also be difficult to quantify C apportionment to a given population within



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the total C mass balance of added substrate due to the challenges of scaling-up results obtained from a few cells.

Therefore, as a response to some of these issues, here we develop a $^{14}C$ labelling and probe-based approach in which FISH-hybridised whole cells are isolated by fluorescence activated cell sorting (FACS) prior to quantification of $^{14}C$ incorporation ($^{14}C$-FISH-FACS). This approach has methodological steps in common with the established technique of MAR-FISH but employs FACS and scintillation counting instead of MAR for high throughput quantification of $^{14}C$ incorporation by hybridised cells. In previous work (Gougoulias and Shaw, 2012), we have evaluated the ability of a Nycodenz -based density gradient centrifugation protocol to obtain representative cell extracts suitable for FACS from soil and also validated the environmental specificity of FISH probe PSE1284 primarily targeting *Pseudomonas* spp. (but also some *Burkholderia* spp.) through 16S rDNA sequencing of FACS-sorted hybridized populations. Accordingly, in this research, we utilised probe PSE1284 and, as a test scenario for the $^{14}C$-FISH-FACS method, chose salicylic acid as the applied carbon source. Salicylic acid is a plant hormone and has been identified as a major root exudate in several plant species (Schmidt et al., 2000; Hao et al., 2010; Khorassani et al., 2011). Members of the *Pseudomonas* genera, the main group targeted here, are known degraders of salicylic acid and also important players in the rhizosphere in consequence of their roles in promotion of plant health and nutrition (Santoyo et al., 2012).



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## Results and discussion

### *Fate of $^{14}C$ salicylic acid*

After an initial short (~4 h) lag phase, salicylic acid underwent rapid mineralization with logistic kinetics that plateaued within 48 hours with a final asymptotic mineralization of ~80% of the $^{14}C$-carbon applied (Fig. 1a). Within the first 24 h, the increase in the mineralized fraction was accompanied by a decrease in the extractable $^{14}C$ from ~70% (at time 0 h) to ~5% of the applied radioactivity (at time 24 h) and a concomitant increase in non-extractable $^{14}C$ associated with the solid phase (post extraction solids (PES); from 33 to ~50%) and extractable biomass (from <0.1 to ~1.6%) $^{14}C$ (Fig. 1b). The rapid dissipation of aqueous-extractable salicylic acid after addition to soil is in agreement with Celis et al. (2005) who, for soil slurry incubations receiving 2.76 or 276 μg $g^{-1}$ salicylic acid, reported a reduction of solution-phase salicylic acid concentration to below detection limits or 40% of that applied initially, respectively, over a 24 hour period. The $^{14}C$ not initially extractable was mostly accounted for in the PES which might be due to rapid sorption of salicylic acid to the soil solid phase, potentially via ligand exchange and formation of bidentate complexes with positively charged metal oxide surfaces (Dubus et al., 2001; Celis et al., 2005; Jagadamma et al., 2012). However, at subsequent time points, the microbial use of salicylic acid C for anabolic (in addition to simultaneous catabolic, i.e. mineralization) processes explains the loss of $^{14}C$ from the aqueous phase and might explain the additional incorporation of $^{14}C$ in to the PES through production of non-extractable microbial products and biomass (Nowak et al., 2011). The microbial metabolism of salicylic acid via catechol or gentisic acid to TCA cycle intermediates has been well studied (Karegoudar and Kim, 2000) and the short lag phase prior to rapid mineralization presumably reflects the time required for the induction of salicylic acid catabolic enzymes (Schell, 1985). The final asymptotic amount of $^{14}C$ mineralized of ~80%



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is in agreement with studies that have characterized the mineralization of low concentrations (1 and 1000 ng $ml^{-1}$) of related aromatic acids (benzoic and phenylacetic) in soil slurries (Amador and Alexander, 1988). The overall mass balance of $^{14}C$ recovered across all fractions varied between 103 ± 1.6 % and 86 ± 0.9 % of total applied radioactivity at 0 h and 12 h (Fig. 1c), respectively; losses due to mineralisation during the Nycodenz extraction processing time (4-6 h) are believed to be responsible for the decreased overall recovery seen at 12h and 24h (Fig. 1c).

*PSE1284-positive population dynamics and salicylic acid C capture*

The PSE1284 hybridising population, which was initially present at ~1.5% of the total FC-detected population increased 7-fold in the 24-h following salicylic acid addition to constitute 9.3 ± 0.2 % of the total FC detected population (Fig 2a and 2b). PSE1284-hybridized populations (n=4 per time point), gated as in the example in Fig. 2a, were recovered by FACS (mean number of cells sorted ranged from 3.7 x $10^5$ to 2.3 x $10^6$, Fig 2b) and the salicylic acid $^{14}C$ associated with the sorted cells quantified by scintillation counting. Examining the apportionment of salicylic acid $^{14}C$ to PSE1284-positive cells over time (Fig. 3) as a function of the $^{14}C$ activity recovered in the total Nycodenz-extracted biomass (also shown in Fig. 1b) reveals an incorporation of 47 ± 4.3% of the salicylic acid-associated radioactivity into the PSE1284-positive biomass over the initial 12 hour period that corresponded to a 4-fold increase in the proportional population size (Fig. 2b). In the subsequent 12 to 24-hour period, PSE1284-positive cells continued to increase their relative abundance (Fig 2b), but their capture of $^{14}C$ as a proportion of the $^{14}C$ initially added as salicylic acid remained relatively static whilst the Nycodenz-extracted biomass as a whole continued to assimilate $^{14}C$ resulting in a decreased



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apportionment of salicylic acid $^{14}C$ to PSE1284-positive cells at 24 hours (33 ± 4.2% $^{14}C$ capture by PSE1284 as a function of the total $^{14}C$ assimilated). This reduced $^{14}C$ apportionment might reflect: (i) succession in the assimilation of the added substrate (the PSE1284 population competitively depleted initially high substrate concentrations prior to the utilisation of residual concentrations by a more oligotrophic population); (ii) the turnover of PSE1284-assimilated salicylate $^{14}C$ and consequent cross-feeding by PSE1284-negative cells. The competitive utilisation of native (potentially primed; Blagodatskaya and Kuzyakov, 2008) soil substrates likely explains how the PSE1284 population could continue to increase in relative abundance in the 12 to 24-hour period (Fig 2b) in the absence of concomitant anabolism of the added $^{14}C$ source.

As already established, probe PSE1284 targets both *Pseudomonas* and *Burkholderia* spp. It is very likely, given the vast microbial phylogenetic biodiversity known to exist in soils (Lundberg et al., 2012; Peiffer et al., 2013; Chen et al., 2016) that the PSE1284-targetted population represented a small proportion of the diversity present in the studied soil. Against this backdrop, that the PSE1284-positive population was initially able to capture nearly 50% of the carbon that was assimilated in to the extracted biomass suggests specificity/selectivity of salicylic acid for the target population. Plasmid- and chromosomally-mediated biodegradation of salicylic acid has long been associated with members of the genus *Pseudomonas* (e.g. Chakraba.Am, 1972; You et al., 1991; Bosch et al., 1999; Nishi et al., 2000; Sazonova et al., 2008), including *Pseudomonas cepacia* (reclassified as *Burkholderia cepacia* complex; Ramsay et al., 1992) and the C apportionment and response of the PSE1284 population to salicylic acid addition here is compatible with earlier reports that salicylic acid might be used as an "exotic" carbon source to



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selectively promote the survival and activity of pseudomonad inoculants in soil (Colbert et al., 1993a; Colbert et al., 1993c; Colbert et al., 1993b). To understand if this selectivity arose because the distribution of the salicylic acid biodegradation trait was conserved within the bacterial groups targeted by the PSE1284 probe, or, through the competitiveness of the PSE1284 population for this substrate against a background of other biodegradative groups, we analysed the taxonomic distribution of putative salicylate hydroxylase enzymes which catalyse the first step in the bacterial degradation of salicylic acid (Supporting Information). This analysis revealed that salicylic acid biodegradation is widely dispersed among bacterial phyla (Supporting Fig. S1) and lower taxomonic levels (Supporting Fig. S2) that are commonly represented in rhizosphere soil and therefore that this trait is not taxonomically conserved. This finding is in agreement with Martiny et al. (2013) who also found that the distribution of traits associated with the assimilation of small organic carbon sources (including p−hydroxy−phenylacetic acid, an aromatic acid) across a range of bacterial phyla to be highly dispersed. Therefore, on the basis of this analysis, it appears that success of the PSE1284 population in exploiting the applied salicylic acid carbon was probably not due to it being the only population in possession of the genetic potential to be able to do so. Pseudomonas spp. and Burkolderia spp. are well known for their adaptive potential (Silby et al., 2011; Vial et al., 2011) and high competitive ability with strategies, in addition to relatively fast growth rates (Cray et al., 2013), that might allow representatives from these two bacterial genera to outcompete others for salicylic acid carbon. Such strategies might include production of various antibiotic compounds (Raaijmakers and Mazzola, 2012), production of siderophores (dos Santos et al., 2004) and siderophore piracy (Galet et al., 2015).



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*Evaluation of the $^{14}$C-FISH-FACS method*

Methods which allow the quantification of C apportionment within the microbial biomass are needed for improved understanding of the relationships between microbial identity, microbial C utilisation traits and C dynamics in soil. Out of the many methods capable of linking biogeochemical processes to specific microbial taxa in the environment, only methods detecting C isotopic incorporation at the whole cell level (rather than biomolecule) level are suitable for the quantification of flows of total substrate carbon through microbial populations. However, as already discussed, current methods which rely on single cell isotopic analysis (by MAR or NanoSIMS) are limited in the number of cells per sample that can be analysed (Clode et al., 2009; Wang et al., 2016). Here, through the application of FACS to sort target cells from the total Nycodenz-extracted cell population we have increased the target cell population that can be analysed from tens of cells to $10^5$ to $10^6$ cells (Fig. 2b) with modest sorting times of ~ 2 hours. Whilst Nycodenz gradient extraction does not likely achieve exhaustive extraction of bacterial cells from soil (Supporting Table S1; Gougoulias and Shaw, 2012), we know from previous work (Gougoulias and Shaw, 2012) that a diverse community is recovered although with some extraction bias. In relation to this, a key assumption of the method as applied to apportion C flow through microbial communities is that any bias in extraction is constant with sampling time and therefore that changes in the proportional abundance of PSE1284-positive cells and in the substrate $^{14}$C they captured are representative of dynamics within the total soil bacterial community.

There have been other published studies that have exploited FACS for the isolation and concentration of marine or lacustrine microbial populations for subsequent isotopic analysis.



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However, these studies have used $^{13}C$ (natural abundance or enriched) and therefore required a custom-built spooling wire microcombustion (SWiM) device coupled to a conventional IRMS (Eek et al., 2007; Hansman and Sessions, 2016) or NanoSIMS (Zimmermann et al., 2015) to detect the $^{12}C/^{13}C$ ratios in the resulting cells as regular combustion elemental analysis-coupled IRMS requires sample masses (10–100 µg C) which are too large to allow analysis of sorted cells (Hansman and Sessions, 2016). In addition, introduction of C-containing fixatives and probes in to cells during FISH protocols might prove problematic in terms of C contamination and therefore for interpretation of sample $^{12}C/^{13}C$ ratios, especially when working at or close to natural abundance levels for $^{13}C$ (Hansman and Sessions, 2016). In comparison to the use of $^{13}C$, the analysis of $^{14}C$ in sorted cell fractions is relatively straightforward using instrumentation (a scintillation counter) that is common place in research laboratories. Because scintillation quantifies $^{14}C$ activity, it is not subject to the same sample mass requirements (or C contamination issues) and therefore allows analysis of a (fixed and hybridised) cell number ($10^5$-$10^6$) that can be realistically achieved by FACS. Here we applied 50 KBq of $^{14}C$-carbon to each experimental unit which resulted in detection of ~ 30-300 Bq $^{14}C$-carbon in the FACS-sorted population over the incubation (Fig. 3) which is ~ 60-600 times the background activity detected in controls not receiving $^{14}C$. This sensitivity allowed detection of $^{14}C$ incorporation and potential cross-feeding over short incubation timecourses (Fig. 3). Whilst we added the test C substrate at a relatively high concentration (50 µg $g^{-1}$) it would be possible to focus on 1000-fold lower concentrations whilst still adding the $^{14}C$ activity required for detection by increasing the specific activity of the substrate applied (based on specific activities of GBq $mmol^{-1}$ that are typical for $^{14}C$-labelled compounds as purchased). Thus, the $^{14}C$-FISH-FACS method can be applied for study of the apportionment of substrates applied at concentrations (ng $g^{-1}$) more



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representative of those found in soil, for example, as a result of root exudation (Jones, 1998), or, indeed potentially to the study of the microbial fate of root $^{14}$C-exudation *in situ*. Furthermore, the $^{14}$C detection in our sorted population of up to ~600 times that of background controls (with 5 min scintillation counting time) points to the capability of the method to be applied to microbial groups that are not such major players in substrate utilisation as the PSE1284 population targeted here. In addition, whilst here we focussed on one PSE1284-positive population, there is the option to target and sort, through the use of FISH probes labelled with different chromophores, multiple bacterial groups.

In terms of the potential limitations of our method, the first relates to the health and safety considerations of working with $^{14}$C and in particular the exposure of workers and environment to $^{14}$C-labelled bacterial aerosol particles during cell sorting. However, our assessment (see Supporting Information) revealed negligible likely exposure from aerosol creation, even under a worst case scenario that simulated a cell sorter nozzle blockage resulting in an unstable sample stream. Secondly, like all probe-based methods, $^{14}$C-FISH-FACS requires prior knowledge of the identity of the population(s) of focus and therefore, on its own, cannot be used as a discovery tool. However, $^{14}$C-FISH-FACS might usefully be combined, in sequence, to learn more about the quantitative C cycling role played by hitherto unknown microbial groups that have been discovered by qualitative identity-function methods such as DNA or RNA-SIP. Thirdly, in comparison to other *in situ* probe-based methods (FISH-MAR, FISH-Raman, NanoSIMS-ISH), the quantification of the isotopic incorporation of the sorted cell fraction in bulk precludes the determination of cell-specific information and therefore analysis of functional differences between single cells of the same population. To overcome this, an aliquot of the sorted cells



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might be taken for subsequent single cell analysis (for example, by MAR) to get an appreciation of the heterogeneity of metabolism within the microbial population of focus.

Balancing the above-discussed advantages and limitations, we conclude that $^{14}$C-FISH-FACS makes a valuable companion to the toolbox of methods capable of quantifying the function of microbial cells *in situ* as demonstrated here in the quantification of the competitive use of salicylic acid by *Pseudomonas* and *Burkholderia* spp. As the Nycodenz protocol used to obtain FACS-ready cell extracts is compatible with monitoring whole system fate (mineralized, extractable, non-extractable) of the added isotope (Fig. 1), including the $^{14}$C incorporated by the extractable non-target as well as the target cell population (Fig. 3) over time, we believe our method allows a quantitative dissection of C flow within the microbial biomass black box that has hitherto not been achieved to the same detail.

## Experimental Procedures

### *Rhizosphere Soil*

A sandy loam soil (2% organic matter, pH 6.5; sampled from the Rowland Series of the University of Reading Farm, Sonning, UK, NGR SU765765) in pots (10 cm diameter containing 270-300 g soil) was sown with *L. perenne* seed (Herbiseed, UK) at a density of 0.013 g seed cm$^{-2}$. After growth (15-20 °C) of the *L. perenne* seedlings for 4 weeks, the whole soil content from triplicate pots (defined as rhizosphere soil) was sieved (<2 mm), mixed to form a composite soil and used for timecourse experiments.



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*$^{14}$C-salicylic acid incubation timecourse*

Replicate gas-tight glass vials (40 ml) containing rhizosphere soil (15.7 g dry weight basis; moisture content 7.8 % v/w) were spiked with a solution of $^{14}$C-salicylic acid (2-hydroxybenzoic acid-phenyl-UL-$^{14}$C) that had been derived from mixing stock solutions of 'hot' $^{14}$C-salicylic acid (≥98% purity, Sigma, UK; dissolved in methanol) and 'cold' sodium-salicylate (dissolved in ultra-pure water) in order to simultaneously deliver 50 µg salicylic acid per gram of soil (dry weight) and 50 KBq of radioactivity per vial. Controls that received only 'cold' salicylic acid (50 µg g$^{-1}$) were included. Small test-tubes containing a solution of NaOH (2M, 2 ml) to trap $^{14}$C-$CO_2$ released as a result of mineralization were placed inside the vials on the soil surface and the vials were sealed and incubated at a constant temperature of 26 ± 0.2 °C, under dark conditions. At 0, 12 and 24 h, four replicate vials were destructively sampled for mineralization: the test tubes were removed and the $^{14}$C in the NaOH quantified by liquid scintillation counting (LSC) using a Wallac 1409 DSA liquid scintillation counter (Perkin-Elmer, Boston, MA) with Ultima-Gold (PerkinElmer) as the scintillant in a 1-to-4 (v/v) sample-to-scintillant ratio. The soil in these vials was then used as the basis for soil extractions as described below. In addition, for extended quantification of mineralization only, replicate vials receiving a lower activity of $^{14}$C-salicylic acid (0.5 KBq) were established for sampling of NaOH traps at time points beyond 24 h.

*Soil extraction, analysis and $^{14}$C mass balance*

The entire soil contents of the vials sampled at 0, 12 and 24 h from the incubation timecourse (above) was slurried in 14.0 ml of phosphate-buffered saline (PBS; 130 mM sodium chloride, 10mM sodium phosphate [pH 7.3]) by vortexing for 2 min with twelve glass beads (6 mm). The



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slurry was then subject to low-speed centrifugation (~600 × g, 5 min, ambient temperature) to sediment large soil particles and debris. The resulting supernatant (containing fine soil particles and microbial cells) was removed and retained, and the slurrying and low-speed centrifugation step was repeated two additional times by adding 8 ml and 7 ml PBS, sequentially, to the remaining soil pellet. The $^{14}$C-activity that remained in the soil pellet (termed Post Extraction Solids $^{14}$C (PES-$^{14}$C)), was quantified by oxidation (four analytical replicates per sample) using a Perkin Elmer Model 307 combustor after freeze drying to avoid any losses of $^{14}$C-salicylic acid due to further mineralisation during drying. The slurry supernatants from the successive extractions were pooled and dispensed to 2 ml microcentrifuge tubes (~10 tubes per sample) and concentrated by high speed centrifugation (16,000 × g, 10 min, 4 $^{o}$C). $^{14}$C-activity in the resulting supernatants ("extractable $^{14}$C") was quantified by LSC as described above for the NaOH samples whilst the pellet (containing fine soil particles and microbial cells) was used as the basis for the Nycodenz purification of bacterial cells (from similar-sized soil particles) to prepare samples ready for FISH, flow cytometry and cell sorting as described below. The total $^{14}$C-activity recovered in each fraction (extractable $^{14}$C, PES-$^{14}$C, bacterial biomass-$^{14}$C) was calculated as a percentage of total applied radioactivity and summed to give the mass balance in each vial. The mass balance included non-biomass associated $^{14}$C that was recovered during the Nycodenz purification, fixation, FISH and washing steps; these fractions were collected, LSC counted and the $^{14}$C activity counted as "extractable $^{14}$C".



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*Nycodenz purification and fixation of bacterial cells*

The main steps of the Nycodenz protocol, as modified from Gougoulias and Shaw (2012) were as follows: the pellets (fine soil particles with bacterial cells) of the initial debris-cleared slurry supernatants were resuspended in PBS (~170 µl per microcentrifuge tube) by rigorous aspiration through a 200 µl pipette tip. These resuspensions were pooled to give 2 × ~850 µl sub-samples which were then layered onto individual density cushions of 1000 µl of a 1.3 g/ml Nycodenz (Gentaur, Belgium) solution. Following centrifugation (16,000 × g, 30 min, 4 °C), the Nycodenz/PBS interface containing the final cell suspension (~400 µl) was removed and diluted twofold with 2 x PBS to provide a purified cell suspension which was harvested by further centrifugation (16,000 × g, 5 min, 4 $^{o}$C). The two sub-samples were combined to form a 300 µl composite sample in 1 x PBS to which an equal volume of 96% (wt/vol) ethanol was added. The fixed samples were stored at -20°C for at least 2 h prior to hybridization and for a maximum period of 2 weeks. An aliquot (20 µl) of the fixed cells was stained with propidium iodide and counted manually under a Brunel epifluorescent microscope to determine the Nycodenz bacterial cell recovery (Supporting Table S1). A further aliquot (30 µl out of 600 µl) of the ethanol fixed cells was directly taken for LSC analysis to quantify the radioactivity associated with the total Nycodenz-extracted bacterial fraction. The steps involved in the soil extraction and Nycodenz purification are depicted diagrammatically in the Supporting Information (Fig. S3).

*Fluorescence in situ hybridization (FISH)*

The following 6-carboxyfluorescein (6-FAM), labeled oligonucleotide probes (Eurofins, MWG-Operon, Ebersberg, Germany) were used: NON-EUB338 [nonspecific for bacteria (Manz et al.,



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1992)] and PSE1284 [designed to target Pseudomonas spp. (Gunasekera et al., 2003)]. Two aliquots (200 µl each) of the remaining ethanol fixed cells were washed twice with 1 ml of PBS and resuspended in 180 µl of prewarmed (48°C) hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.5, 30% formamide, 0.1% sodium dodecyl sulfate) and mixed with 20 µl either of the specific probe PSE1284 or non-specific probe NONEUB-338 (final concentration: 5 ng $\mu l^{-1}$ for each probe). After incubation for 4 h at 48 $^{o}$C, the hybridization mixtures were centrifuged (16,000 × g, 5 min), the cell pellets resuspended in 500 µl of hybridization buffer and incubated at 48 $^{o}$C for a further 20 min. The cells were then harvested by centrifugation and incubated in 500 µl of wash buffer (20 mM Tris-HCl, pH 8.0, 0.9 mM NaCl, 0.1% sodium dodecyl sulfate) for 20 min. Finally, the cells were again harvested by centrifugation and resuspended in 0.2 ml of cold filter-sterilized (0.2 µm) PBS to await flow cytometric analysis and cell sorting.

*Flow cytometry and cell sorting*

Flow cytometric analysis and cell sorting were performed with an Influx™ (Becton Dickinson, formerly Cytopeia Inc, Seattle, USA) flow cytometer/cell sorter equipped with a 488-nm solid state laser (CoherentTM-Sapphire, output power 200 mW) as described previously (Gougoulias and Shaw, 2012). The Influx™ was also equipped with an extra forward-small size particle detector which allows the detection of particles >0.1 µm and was calibrated with 0.53 µm and 3.00 µm fluorescent microbeads (Saxon Europe Ltd, Kelso, UK). The Influx™ data were acquired as pulse height signals for 100,000 events at a rate of 10,000 to 25,000 events per second with the use of the SpigotTM v.6.1.4 (BD, San Jose-California, USA) software and ~370,000 to 2.3 million events related to the PSE-targeted population were sorted per sample and



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LSC counted. Different replicates for each time point were analysed on different days to account for operational variation. During sorting the following parameters were chosen and/or calculated: sheath pressure - 31.7 psi using a 70 µm tip, trigger channel - paraller (small size particle detector), trigger level - 30; drop frequency - 67.9 kHz; piezo amplitude - 2.37-2.51 V; drop delay - 43.8; and, break off point (camera position on drop formation) - 223. Data analysis and graphics were acquired using the FlowJo software package (FlowJo 7.2.4, Tree Star Inc, Ashland, USA).

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**Figure Legends**

**Figure 1**. (a) Cumulative mineralization of $^{14}$C-salicylic acid (50 µg g$^{-1}$) added to soil sampled from the *L. perenne* rhizosphere over a 132 h timecourse. (b) Partitioning the fate of total applied salicylic acid $^{14}$C between mineralized, extractable, post extraction solids (PES) and extracted biomass fractions for the 0-24 hour time points. (c) Mass balance of $^{14}$C recovered across all fractions in (b) for the 0-24 hour time points. Data are mean ± SE (N=4). The 'extractable' fraction in the mass balance includes $^{14}$C recovered by PBS extraction and in washings during subsequent Nycodenz cell extraction and fixation steps. The following functional form was fitted ($p$=0.0003; $R^2 = 0.98$) to the cumulative mineralization data in (a): $Y = a[1 + (t/t_0)^b]^{-1}$ where Y = cumulative percentage of salicylic acid $^{14}$C evolved as $^{14}CO_2$ and t = time. This function has an asymptote at a = 80.8 ± 5%.

**Figure 2.** Flow cytometric (FC) analysis of the response of the PSE1284-6FAM-hybridising population to the addition of salicylic acid (50 µg g$^{-1}$) to *L. perenne* rhizosphere soil. (a) Indicative flow cytometric dot plots where *x* axis = side light scatter and *y* axis = green fluorescence per cell in the FITC channel for fixed cells extracted from soil (i,ii) 0-h, (iii) 12-h and (iv) 24-h after salicylic acid addition and targeted with (i) non-specific probe (NON-EUB338-6FAM,) and (ii-iv) probe PSE1284-6FAM. Plots contain 100,000 events. The gated part of the plots indicates the area where the events were sorted from with the numerical value inside the gate depicting the percentage of the total detected events that are in the gate. Graphs shown are indicative of 4 replicates per time point. (b) Mean (n =4; ± SE) response of the PSE1284-6FAM-hybridising population to the addition of salicylic acid over time (0 -24 h). Data points represent the number of PSE1284-6FAM positive cells as a proportion of the total number of FC detected events (i,e, 100,000 events) whilst the actual number of events sorted as



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PSE1284-6FAM positive (the whole aliquot was sorted until the sample was exhausted) are shown above the data points. The correlation between the percentage PSE1284-detected population by FC and the PSE1284-sorted events by FACS had an $R^2$ >0.99, $p$ <0.001).

**Figure 3**. Apportionment of salicylic acid $^{14}C$ to total Nycodenz extracted and PSE1284-sorted bacterial cells over time following addition of $^{14}C$-labelled salicylic acid (50 ug/g) to soil. Actual amounts of recovered radioactivity (Bq) are shown on the right Y axis (50,000 Bq was spiked per sample of 15.67 g soil dw). Data are mean ± SE. Mean values within fraction with a letter in common are not significantly different ($p$>0.05; Fisher LSD).



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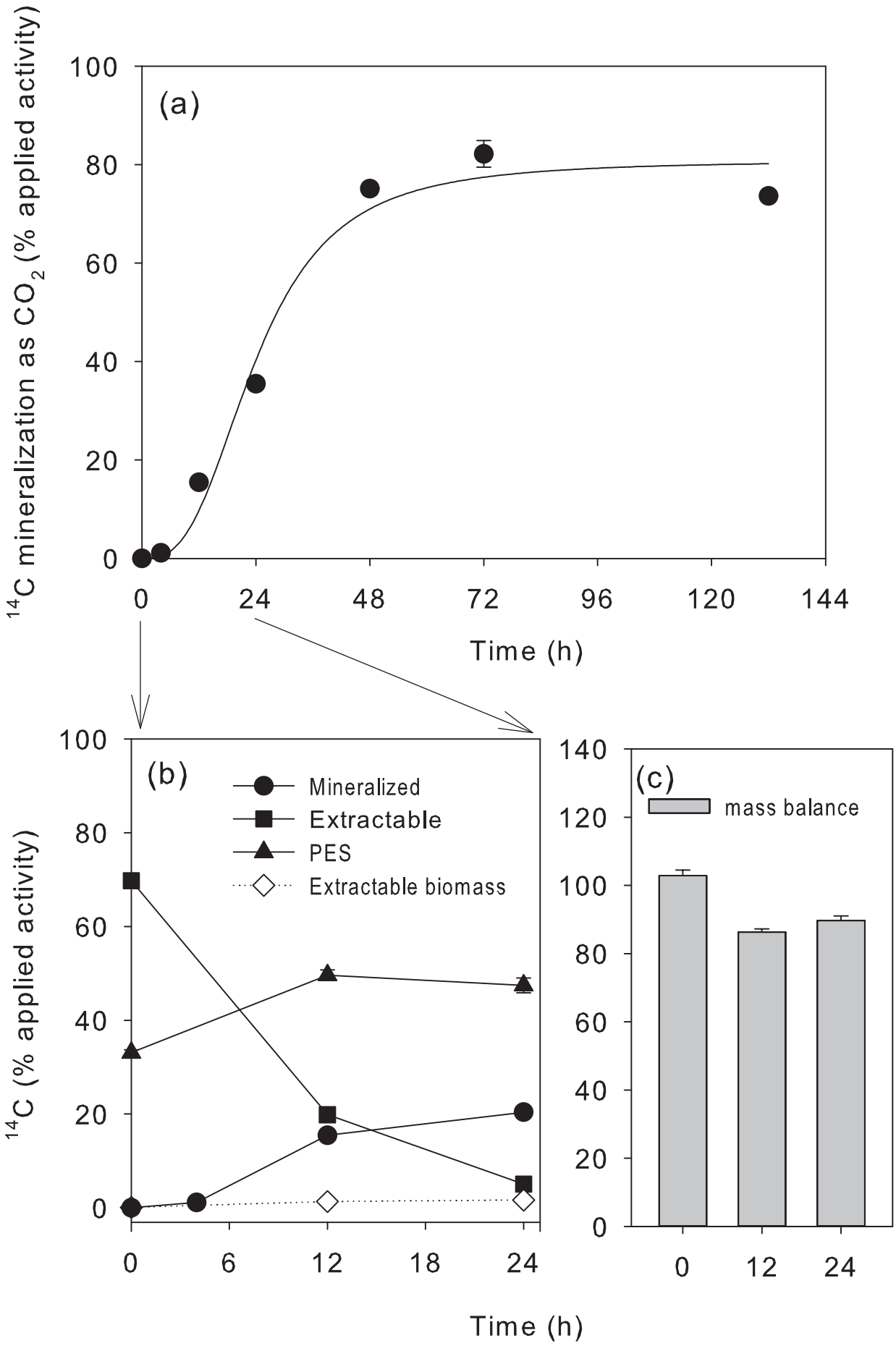


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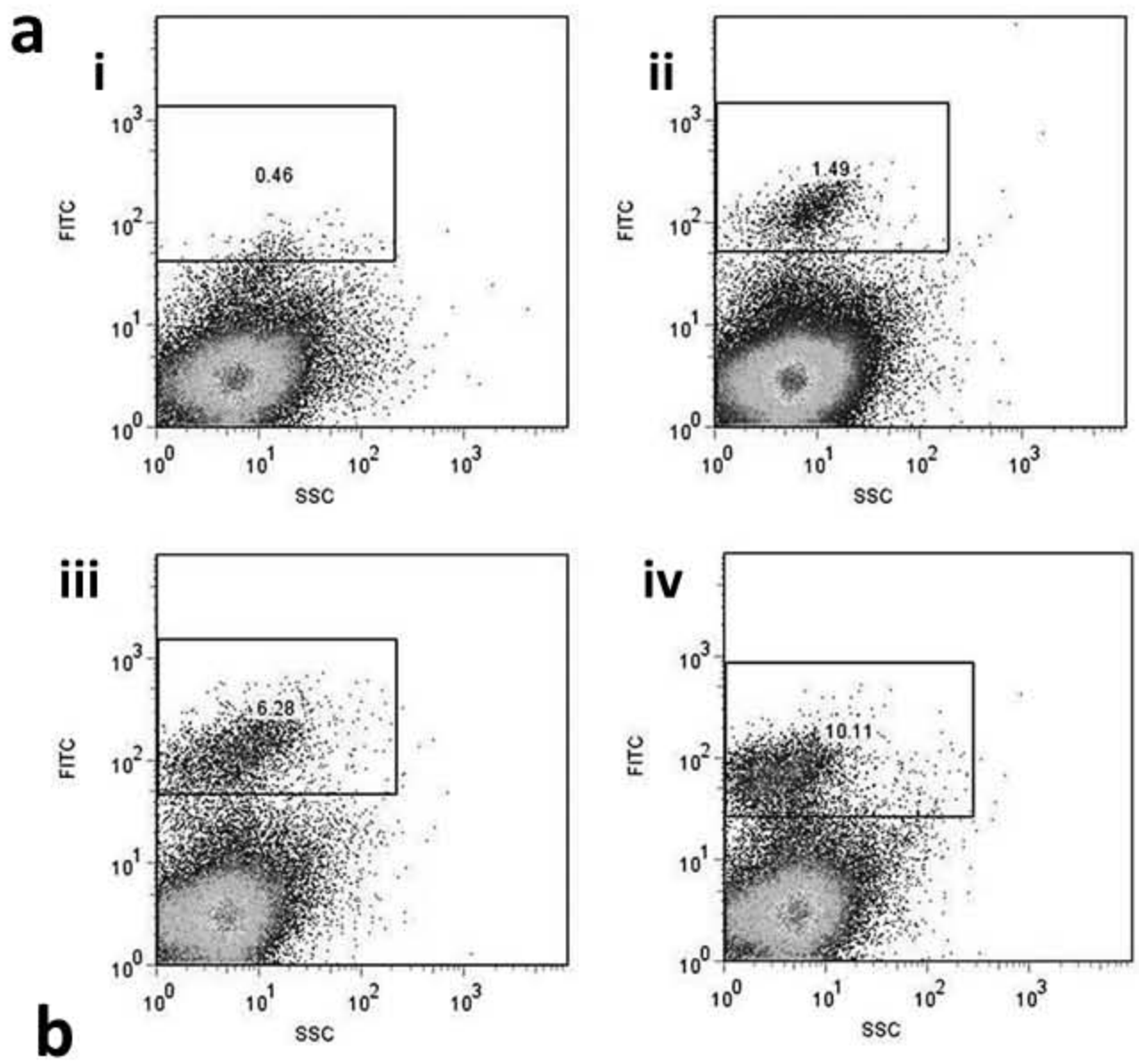


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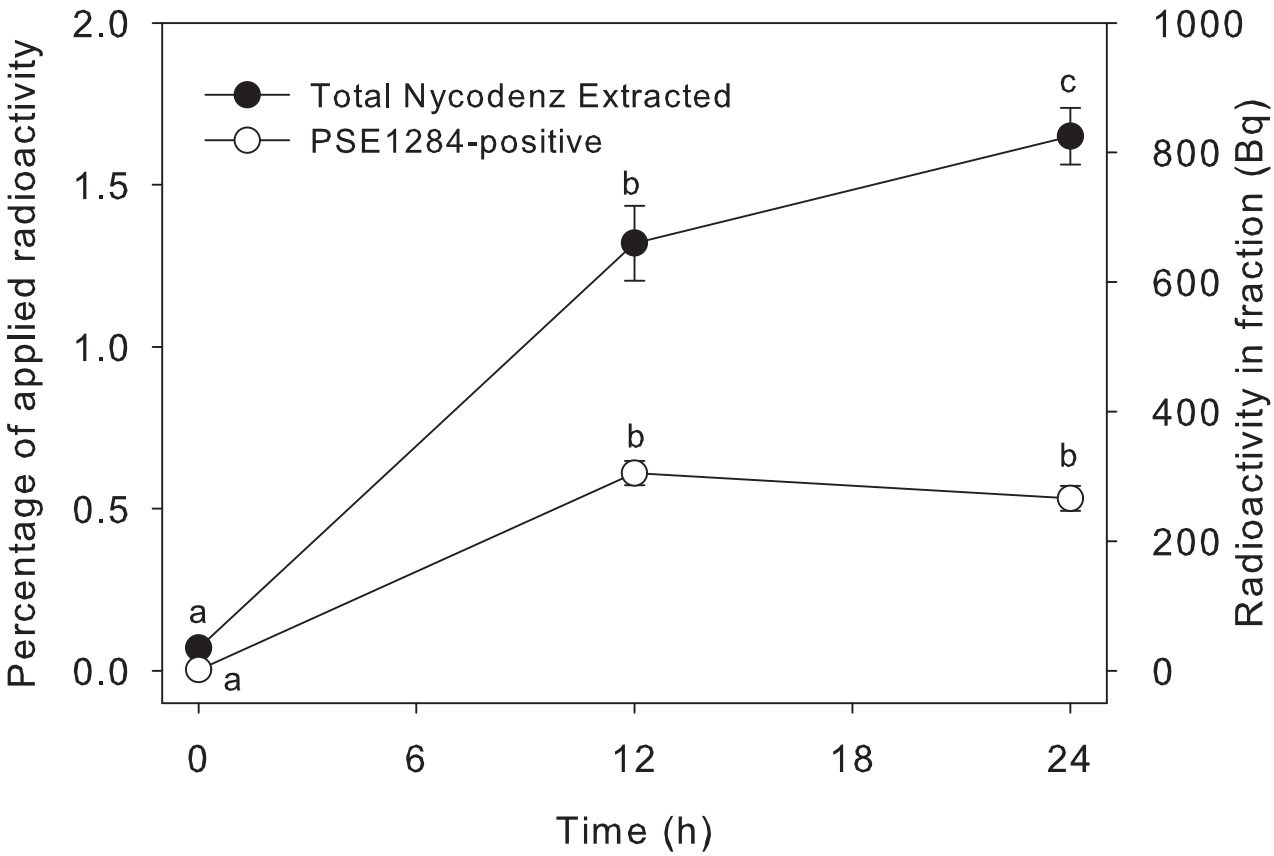


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