

Methyl selenol as precursor in selenite reduction to Se/S species by methane-oxidizing bacteria

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Originally published:

November 2019

Applied and Environmental Microbiology 85(2019)22, e01379-19

DOI: <https://doi.org/10.1128/aem.01379-19>

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1 **Selenite Reduction by Methane-oxidizing Bacteria: Methyl Selenol as Precursor for**
2 **Methylated Se/S Species and Characterization of Se_{8-x}S_x Nanoparticles**

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16 **Summary**

17 A wide range of microorganisms have been shown to transform selenium-containing
18 oxyanions to reduced forms of the element, particularly selenium-containing nanoparticles.
19 Such reactions are promising for detoxification of environmental contamination and
20 production of valuable selenium-containing products such as nanoparticles for application in
21 biotechnology. It has previously been shown that aerobic methane-oxidising bacteria,
22 including *Methylococcus capsulatus* (Bath), are able to perform methane-driven conversion
23 of selenite (SeO₃²⁻) to selenium-containing nanoparticles and methylated selenium species.
24 Here, the biotransformation of selenite by *Mc. capsulatus* (Bath) has been studied in detail

25 via a range of imaging, chromatographic and spectroscopic techniques. The results indicate
26 that the nanoparticles are produced extracellularly and have a composition distinct from
27 nanoparticles previously observed from other organisms. The spectroscopic data from the
28 methanotroph-derived nanoparticles are best accounted for by a bulk structure composed
29 primarily of octameric rings in the form $\text{Se}_{8-x}\text{S}_x$ with an outer coat of cell-derived
30 biomacromolecules. Among a range of volatile methylated selenium and selenium-sulfur
31 species detected, methyl selenol (CH_3SeH) was found only when selenite was the starting
32 material, although selenium nanoparticles (both biogenic and chemically produced) could be
33 transformed into other methylated selenium species. This result is consistent with methyl
34 selenol being an intermediate in methanotroph-mediated biotransformation of selenium to all
35 the methylated and particulate products observed.

36 **Keywords:** Selenite reduction, Elemental selenium, Methane-oxidizing bacteria, Mixed
37 chalcogenide amorphous nanoparticles

38 **Introduction**

39 A key biotransformation mechanism of most microorganisms exposed to selenium oxyanions
40 is dissimilatory reduction to nanoparticulate elemental selenium (Lortie *et al.*, 1992; Kessi *et al.*,
41 *et al.*, 1999; Prakash *et al.*, 2009; Nancharaiah & Lens, 2015b). The formation of the
42 nanoparticles (NPs) reduces the toxicity and bioavailability of the selenium species (Combs *et al.*,
43 *et al.*, 1996; Nancharaiah & Lens, 2015a; Eswayah *et al.*, 2016; Song *et al.*, 2017; Vogel *et al.*,
44 2018). Not only does the formation of the NPs reduce the adverse environmental impact of
45 the oxyanions on the microorganisms and their surroundings but present an approach that can
46 potentially be harnessed to produce selenium NPs tailor-made for a variety of technological,
47 clinical, analytical and industrial applications (Prasad, 2009; T. Wang *et al.*, 2010; Bai *et al.*,
48 2011; Tian *et al.*, 2012; Iranifam *et al.*, 2013; Ren *et al.*, 2013; Tran & Webster, 2013; J.
49 Wang *et al.*, 2014; Ramya *et al.*, 2015; Jain *et al.*, 2016; Wadhvani *et al.*, 2016). However,
50 made-to-order NPs with microbial intervention can only be achieved when the structural
51 features of the NPs produced by the different bacteria are better understood and characterised.

52

53 In their 2004 paper, Oremland et al investigated the structural features of selenium
54 nanospheres produced by Se-respiring bacteria (Oremland *et al.*, 2004). They found that the
55 three bacteria studied produced red amorphous Se NPs with distinct features, two contained
56 predominantly Se₆ chain units, and the third had Se₈ ring units. Hu and Barton found in an
57 investigation into the reduction of selenium oxyanions by *Desulfovibrio desulfuricans*, a
58 sulfate-reducing bacterium, that amorphous spherical submicro particles containing selenium
59 and sulfur were produced both inside and outside the cell. They found that the bacterium was
60 more effective at reducing selenite. The authors also proposed the mechanisms by which the
61 particles were formed (Hu and Barton, 2013). More recently, Vogel et al proposed that the
62 biogenic selenium NPs produced by *Azospirillum brasilense* from the biotransformation of
63 selenite is cyclic Se_{8-n}S_n with Se₆S₂ the most likely structure (Vogel *et al.*, 2018). Tugarova et
64 al reported the characterisation of amorphous selenium particles produced by *Azospirillum*
65 *thiophilum* and found the presence of only selenium with no evidence of either Se-S or S-S
66 bands in the Raman spectrum (Tugarova *et al.*, 2017). Moreover, Ruiz-Fresneda et al (2018),
67 described the ability of *Stenotrophomonas bentonitica* for biogenic reduction of Se(IV),
68 production of amorphous Se⁰ (a-Se) nanospheres and their subsequent transformation to one-
69 dimensional (1D) trigonal selenium (t-Se) nanostructure where sulfur was associated with the
70 SeNPs. In our study, Eswayah et al, we presented indirect evidence from transmission electron
71 microscopy (TEM) imaging with energy dispersive X-ray spectrometry (EDX) measurements
72 to show that sulfur was associated with amorphous selenium in the extracellular NPs that are
73 produced from the reduction of selenite by methane-oxidizing bacteria (Eswayah *et al.*, 2017).

74
75 Besides the above-mentioned studies on the biotransformation of selenium oxyanions most
76 investigations have focused on the formation of the NPs. A few others have identified
77 concomitant release of volatile selenium species into the headspace gas (Chasteen, 1993;
78 Burra *et al.*, 2010). However, none of these approaches has provided enough information to
79 enable the elucidation of the processes leading to the formation of the amorphous NPs.
80 Indeed, the formation of the extracellular amorphous NPs forms as reported in many studies
81 (Oremland *et al.*, 2004; T. Wang *et al.*, 2010; Dhanjal & Cameotra, 2010; Zhang *et al.*, 2012;
82 Kamnev *et al.*, 2017) may indicate limited direct involvement of the microorganisms in their
83 formation. In our experiments, we observed the size of the extracellular nanoparticles
84 increases with time, which suggests that the growth of the NPs is a result of abiotic reactions
85 in the culture medium outside the cells. The formation of mixed chalcogenide species by

86 exchange reactions, when both Se and S species are present in the gaseous and solution phase,
87 has been reported (Meija & Caruso, 2004; Vriens *et al.*, 2015). It is probable that similar
88 reactions occur in the culture medium solution resulting eventually to the formation of the
89 nanoparticles.

90 In order to gain a better understanding of the biotic and abiotic transformations occurring in
91 the culture, both selenium- and sulfur-containing species were sampled from the headspace
92 and solution of selenite amended and control samples at fixed times by sorptive extraction in
93 conjunction with analysis by thermal desorption - gas chromatography- mass spectrometry
94 (TD-GC-MS) to identify the compounds. In parallel, the formed NPs were characterised by a
95 range of physical techniques, namely; attenuated total reflectance Fourier transformation
96 infrared spectroscopy (ATR-FTIR), Raman spectroscopy, transmission electron microscopy
97 (TEM) and energy dispersive X-ray (EDX) spectrometry, X-ray absorption spectroscopy
98 (XAS), and X-ray photoelectron spectroscopy (XPS). Herein, the results obtained from these
99 measurements are used to inform the formation and elucidation of the structure of the sulfur-
100 doped red amorphous selenium NPs produced when the methane-oxidizing bacterium
101 *Methylococcus capsulatus* (Bath) reduces selenite.

102 **Results**

103 Preliminary investigations with the methanotroph species *Mc.capsulatus* (Bath) showed that
104 the sizes of the NPs grew rapidly from an average of 220 ± 51 nm in the first 4 hrs to about 400
105 ± 77 nm in the next 44 hrs as previously published (Eswayah *et al.*, 2017). It is evident from
106 the HAADF-STEM imaging and TEM thin-section micrographs of the nanoparticles produced
107 by the bacterium that the NPs are associated extracellularly with the cells (see Fig. 1a).
108 Furthermore, Se and S distributions in the EDX maps overlap, indicative of a spatial and likely
109 structural association, suggesting the formation of mixed chalcogenide nanoparticles (see
110 Fig.1b). The intensity of the Se signals was, however, much higher than that for S (see Fig.
111 1c). In addition, examination of the $S_{k\alpha}$ map reveals that not only was there sulfur in the
112 particles but there was a trail of the element linking the particles to likely sulfur-containing
113 proteins from the bacterial cells.

114 Se K-edge X-ray Absorption Near-Edge Structure (XANES) spectra of the particles formed
115 by *Mc. capsulatus* (not shown) are in line with those of red amorphous Se with no detectable
116 residual selenite present in the samples. Shell fitting of the Extended X-ray Absorption Fine-

117 Structure (EXAFS) spectra showed the characteristics of red elemental Se⁰, with a Se-Se path
118 at a distance of 2.35Å. A second Se-Se shell was detected at 3.69Å with coordination
119 numbers less than what has been previously been observed for amorphous Se (Scheinost &
120 Charlet, 2008; Scheinost *et al.*, 2008), possible indication for the presence of a mixed Se/S
121 phase. This small coordination number cannot be attributed to the NP size far above 10 nm,
122 nor to a high structural disorder, the Debye-Waller factor (σ^2) of this second Se-Se showed
123 increasing structural order with particle growth over time, without increasing the respective
124 coordination numbers (see Eswayah et al (2017) for a detailed discussion of these results).
125 The small coordination numbers hence suggest the presence of (more weakly backscattering)
126 S atoms, but attempts to establish their presence by shell fitting failed because of strong
127 destructive interference between Se-Se and Se-S paths.

128 In order to unravel the make-up of the nanoparticles, the surfaces of the particles were
129 characterized by FTIR, XPS and Raman spectroscopy.

130 **FTIR Analysis**

131 The FTIR spectra of the freeze-dried selenium nanoparticles produced by the *Mc. capsulatus*
132 in liquid NMS medium amended with selenite, the Chem-NPs and the bacterial biomass are
133 shown in Figure 2. The assigned bands are summarised in Table 1.

134 **XPS analysis of the particle surface**

135 The surface composition of the harvested red particles was obtained by XPS, and the
136 elemental content are summarised in Figure 3a. In addition to the selenium which is present at
137 a concentration of 1.25 Atomic weight%, there are five other elements: carbon (46.32%),
138 oxygen (31.41%), nitrogen (8.61%), calcium (5.89%) and phosphorus (4.77%) that were
139 detected on the surface of the particles. The presence of the first three elements is an
140 indication that there are organic molecules on the particle surfaces. The high resolution
141 spectra scans for Se, C, N, and O, and the assigned chemical species from the core level XPS
142 spectra of C1s, N 1s, O 1s bands are shown in Figures 3b-e. The spectrum for 3d Se shows a
143 doublet which is unresolved. However, the fitted deconvoluted peaks show two predominant
144 bands at 55.16 and 56.02 binding energy (eV), and two minor peaks at 55.75 and 56.61 eV,
145 respectively, the later pair of peaks resulting from the Se 3d peak split by spin orbit coupling
146 into Se 3d_{5/2} and Se 3d_{3/2}. The observed range of binding energies between 55.16 and

147 56.61eV is indicative of the presence of reduced selenium species, including elemental
148 selenium (Naveau *et al.*, 2007).

149 **Raman characterisation of the amorphous particles**

150 Vibrational spectroscopy particularly Raman spectroscopy has been the technique of choice
151 for the characterisation of Se_n allotropes, and aggregates. The deconvoluted spectrum
152 obtained between 50-600 cm⁻¹ Raman shift from the harvested SeNPs is shown in Fig 4.
153 There are four bands which are visible: the main band at 251.5 cm⁻¹ and smaller ones at 80.2,
154 358.8 and 506.5 cm⁻¹, respectively. All of the bands were present in all of the scans of
155 samples collected at different time points: 6, 24 and 48h. The band at 513.5 cm⁻¹ was more
156 prominent than that at 358.8 cm⁻¹. The band at 80.2 cm⁻¹, which is a shoulder, is only visible
157 in the deconvoluted spectrum.

158 **Speciation of selenium and sulfur in the medium solution and headspace**

159 The GC-MS chromatograms of the species found in both the headspace and solution of the
160 selenite amended medium at 4h and 20h are shown in Figures 5 and 6, respectively. The
161 earlier time was chosen because the formation of the particles and therefore the red colour of
162 the solution were barely discernible. A summary of the selenium- and sulfur-containing
163 species are given in Table 3. Examination of the data in the Tables showed that after 4h, three
164 compounds: methyl selenol (MSeH), dimethyl selenenyl sulfide (DMSeS) and dimethyl
165 diselenide (DMDS_e) were detected in both the solution and headspace. In addition dimethyl
166 diselenenyl sulfide (DMDS_eS) was also found in the solution. At 20h, three new species:
167 Bis(methylselenomethane) (DMSe), dimethyl selenenyl disulfide (DMSeDS), and dimethyl
168 diselenenyl sulfide (DMDS_eS) were detected in the solution in addition to triselenothone/
169 dimethyltriselenide (DMTSe) which was detected in both the solution and headspace.

170 **Discussion**

171 The results from the kinetics experiments showed that there were increases in particles sizes
172 with incubation time leading us to hypothesize that much of the structure of the particles was
173 formed in the extracellular space. If this is true, then the key reactions resulting in the
174 increase in the particle sizes are essentially abiotic in nature. Consequently, the clues to the
175 structural formation of the particles must lie in the nature and identity of the compounds that
176 are concomitant in the solution and headspace of the nascent particles. It was therefore

177 essential to sample for selenium- and sulfur-containing compounds in both the headspace and
178 solution, followed by their analyses and identification.

179 The FTIR spectra of the SeNPs produced by *Mc. capsulatus*, samples of biomass of the strain
180 (control), as well as the Chem-SeNPs were recorded in order to identify the functional groups
181 capping the synthesized SeNPs. The peak centred at 3297 cm^{-1} corresponds to the -OH and
182 -NH stretching vibrations of the amine and carboxylic groups. Peaks at 2927 cm^{-1}
183 corresponded to the aliphatic saturated C-H stretching modes (Naumann *et al.* 1995; Kamnev
184 *et al.* 2017). The peaks at 1644 , 1538 , and 1239 cm^{-1} are characteristic of amide I, amide II,
185 and amide III bands of proteins, respectively (Alvarez-Ordóñez *et al.* 2011; Ojeda & Dittrich
186 2012). The symmetrical stretch of carboxylate group can be attributed to the bands observed at
187 1366 cm^{-1} . The peaks at 1150 , 1077 and 1015 cm^{-1} corresponded to the C-O stretching
188 vibrations of C-O-C groups (Naumann *et al.* 1995; Beekes *et al.* 2007). The presence of
189 phosphoryl groups was confirmed by the peak at 919 cm^{-1} . Additionally, peaks at 859 and 762
190 cm^{-1} (fingerprint region) could be mainly attributed to aromatic ring vibrations of aromatic
191 amino acids (tyrosine, tryptophan, phenylalanine) and possibly nucleotides (Burattini *et al.*
192 2008; Kamnev 2008).

193 The FTIR spectra of SeNPs of *Mc. capsulatus* differ from those of the bacterial biomass
194 (control) and the Chem-SeNPs. The main difference between the spectra is that the Bio-
195 SeNPs exhibit more peaks in the protein and polysaccharide vibration region, indicating the
196 presence of proteins and polysaccharides in the biomacromolecules capping the SeNPs
197 (Shirsat *et al.*, 2015; Wadhvani *et al.*, 2016; Tugarova & Kamnev, 2017; Kamnev *et al.*,
198 2017).

199 By contrast, Chem-SeNPs obtained through reaction of Na_2SeO_3 with L-cysteine displayed a
200 broad absorption band around 3350 cm^{-1} and absorption band at 2923 cm^{-1} that are assigned
201 to O-H vibrations of the absorbed H_2O and C-H vibration in the alkyl chain of L-cys,
202 respectively. The peak at 1606 cm^{-1} can be mainly attributed to C=O vibrations. It is
203 noteworthy that the presence of organic residues such as carbohydrates, lipids, and proteins on
204 the surface of biogenic SeNPs were completely absent in the Chem-SeNPs spectrum (see
205 Figure 2) FTIR spectra of the Bio-SeNPs separated from the *Mc. capsulatus* cells showed
206 bands typical of proteins, polysaccharides and lipids associated with the particles (in line with
207 their TEM images showing a thin layer over the particles), in addition to strong carboxylate
208 bands, which may stabilise the SeNPs structure and morphology.

209 The XPS results show two Se containing species, and other organic constituents.

210 In assigning the Se bands, it is essential to link the structure of the particles to the selenium-
211 containing species that have been identified in the solution (see the discussion of the TD-GC-
212 MS results below). Since data for the exact compounds are not available, structures that may
213 be similar to these in the particles have been selected. The major band at 55.16 eV, has been
214 assigned to the compound: $(\text{CH}_3)_2\text{NC}(\text{Se})\text{SeC}(\text{Se})\text{N}(\text{CH}_3)_2$ (Kobayashi *et al.*, 1986). This is a
215 reasonable fit to the results obtained in this study not only because of the presence of selenium
216 but also the content of the methyl groups and nitrogen. The nitrogen could account for the
217 single band at 400.10eV usually assigned to amine nitrogen. The bands at 56.02 eV has been
218 assigned to $(-\text{CSeC}(\text{CH}_3)\text{C}(\text{CH}_3)\text{Se}-)_2$ (Dáaz *et al.*, 1996). Both of these assignments are an
219 indication of the presence of long chain of selenium-containing methylated species. Missing
220 from both spectra is oxygen which is presumably present as C=O, and either C-O-C or C-O-H
221 at 531.58 and 532.84 eV, respectively.

222 However, the presence of Se-C was not detected in the spectrum thus indicating the amount
223 of carbon directly bound to selenium was low. Furthermore, because of the high
224 concentration of selenium in relation to sulfur, the signal for the latter could not be resolved
225 and identified.

226 Whereas, both FTIR and XPS provided information on the forms in which the elements are
227 present on the particle surface, Raman spectroscopy enables the identification of the basic
228 structural make-up of the particles. It has been proposed that amorphous selenium is composed
229 of a mixture of Se_n rings and helicoidal chains (Lucovsky *et al.*, 1967; Yannopoulos &
230 Andrikopoulos, 2004; Demchenko *et al.*, 2010). The proportion of each depends on chemical
231 and physical conditions under which the samples are made, and the treatments to which they
232 have been subjected. According to Carini et al the band at around 250 cm^{-1} is characteristic of
233 amorphous selenium (Carini *et al.*, 1980). The symmetrical band at 251.5 cm^{-1} , with full width
234 half maxima of 30 cm^{-1} found in this study is characteristic of Se-Se stretching vibration in
235 pure Se_8 (Lucovsky *et al.*, 1967; Baganich *et al.*, 1991; Nagels *et al.*, 1995) with its'
236 deformation vibration at the deconvoluted band of 80.2 cm^{-1} . However, in the study of mixed
237 selenium and sulfur alloys by Machado et al, the authors state that as the sulfur concentration
238 increases in mixed amorphous selenium, the peak at 234 cm^{-1} usually associated with Se
239 chains decreases in intensity and the band at 250 cm^{-1} , usually associated with selenium rings
240 increases in intensity. They also observed the appearance of a band at

241 352 cm^{-1} as the selenium to sulfur ratio increases to either 4:1 or 7:3 (Machado *et al.*, 2010).
242 The band at 358.8 cm^{-1} has been assigned to S-Se stretching vibration (Eysel & Sunder, 1979;
243 Kasuya *et al.*, 1996). The band at 513.5 cm^{-1} is probably due to the Se-Se overtone band of
244 the fundamental band at 251.5 cm^{-1} . An important observation in this regard is that the S-S
245 vibration band is not seen when the S composition is below that found in Se_6S_2 (Lucovsky *et al.*
246 *et al.*, 1967). Therefore it is probable that the composition of the harvested particles is $\text{Se}_{8-x}\text{S}_x$,
247 where x is equal to or greater than 2.

248 The intensity of the band 251.5 cm^{-1} is indicative of the predominance of the Se-Se bonds in
249 the structure of the particles. It is probable that S is integral to the mixed particle structure
250 $\text{Se}_{8-x}\text{S}_x$, and not as an S_8 impurity in the particles. The low available sulfur content in the
251 medium makes the formation of an S-S homonuclear bond in the mixed particle structure
252 highly unlikely (Eysel & Sunder, 1979).

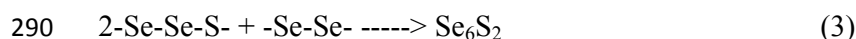
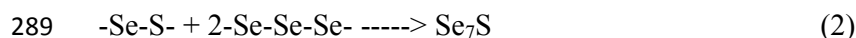
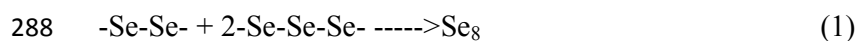
253 We have previously reported that methyl selenol is produced and detected in the head space
254 only when selenite is in the starting medium (Eswayah *et al.*, 2017). Besides the presence of
255 methyl selenol in the headspace, methyl selenoacetate was also detected. However, in the
256 present study methyl selenoacetate was not detected when the headspace sorptive extraction
257 probes were deployed for sample collection. No methyl selenol was detected when either the
258 harvested or chemically synthesized nanoparticles were added to the medium in the absence
259 of selenite. Therefore, we propose that methyl selenol may be the precursor of all of the
260 methylated selenium species as well as the selenium-containing nanoparticles. If this is the
261 case, the first step in the biotransformation of selenite would involve the reduction and
262 methylation of selenite to methyl selenol followed by the formation of the other selenium-
263 and sulfur-containing species. Indeed, the formation of some of the latter species requires the
264 presence of the nascent selenium particles.

265 Based on these observations a possible pathway for the formation of the particles and the
266 other products of biotransformation of selenite can be outline as in Scheme 1.

267 A series of abiotic reactions proposed by Ganther (1968; 1971) and outlined by Xu and Barton
268 (2013) implicate glutathione (GSH) and GSH reductase in the production of elemental
269 selenium. The proposed steps leading up to the formation of methyl selenol are shown in
270 Scheme 2.

271 The presence of sulfur-containing species was detected in the headspace and culture
 272 supernatant of the control *Mc. capsulatus* culture without added selenite. These species
 273 included: benzothiazole, dodecanethiol, and propanesulfonyl, which are the likely source of
 274 sulfur in the structure of the particles. Evidence of the formation of the longer chains of the
 275 selenium- containing species can be seen in the nature of the compounds that are found in the
 276 solution after hours of incubation, first after 4h, dimethyl diselenenyl sulfide was detected,
 277 and subsequently dimethylselenodisulfide, dimethyltriselenide and bis(methylseleno)
 278 methane were detected after 20h. More complex mixed Se and S compounds are formed in
 279 the medium with time. The formation of the mixed chalcogenides of Se and S is hardly
 280 surprising since S may be available from the reduction of sulfate in the growth medium. A
 281 key question therefore is how the solution chemistry relates to the observed structural
 282 features of the Se particulates.

283 It is likely that these longer chains polymerize to form Se_x or $Se_{8-x}S_x$ linear or cyclic
 284 structures. Indeed, all chalcogen elements have the tendency to form cyclic allotropes. The
 285 dominant allotrope will depend on the experimental conditions. Examples of exchange
 286 reactions that may occur based on the presence of the detected selenium- and sulfur-
 287 containing species are shown in the following equations:



291 As can be seen a variety of nanocomposites can be formed. Similar exchange reactions have
 292 been shown to occur when mixed Se and S complexes are present in the same solution
 293 (Vriens *et al.*, 2015). Indeed these reactions are known to occur in amorphous selenium
 294 semiconductors (Stuedel, 1986). The Raman results indicate these are not open chains
 295 clusters but cyclic structures with the Se_8 structure dominant and the probable presence of
 296 small amounts of Se-S bonds. To prevent the introduction of sulfur into the structures, the
 297 presence of the element could be reduced from amount in the culture medium if this does not
 298 affect the bacterial growth.

299 The formed particles are surrounded and stabilized by the presence of polymeric substances
 300 as proposed by Jain *et al* (2015) as evidenced by the XPS and FTIR measurements.

301 In this study, we demonstrate for the first time that in the reduction of selenite by *Mc.*
302 *capsulatus* (Bath), a methane oxidizing bacterium, methyl selenol is the likely precursor for
303 the formation of methylated selenium-containing and mixed chalcogenides species.
304 Subsequent exchange reactions between the species result in the formation of the amorphous
305 allotropic form of selenium, cyclic Se₈ with sulfur in its structure. The nature of the molecular
306 mediators in reduction of selenite, supplying sulfur that is integral to the structure of the
307 nanoparticles and supplying the methyl groups found in the volatile selenium containing
308 products remain to be identified.

309 **Experimental Procedures**

310 **Bacterial strains and growth conditions**

311 The methanotrophic bacterium *Methylococcus capsulatus* (Bath) (NCIBM 11132) was grown
312 and propagated aerobically using methane as the carbon and energy source as previously
313 described (Eswayah et al, 2017). For these experiments the initial selenite concentration used
314 was 20 mg L⁻¹.

315 **Detection of solution and volatile selenium species**

316 Solution and volatile selenium-containing species were sampled by immersive sorptive
317 extraction using sampling probes (HiSorb probe, Markes International, UK) from either the
318 solution or headspace. Extension screw-on arms were fabricated for each probe so that they
319 could be inserted through the Suba-Seals used to seal the necks of the culture flasks. To
320 ensure that the probes and tubes were contamination free, before use, the probes and tubes
321 were preconditioned with helium at flow rate of 90 mL min⁻¹ using the following temperature
322 programme: 15 min at 100 °C, 15 min at 200 °C, 15 min at 300 °C and 15 min at 335 °C. The
323 preconditioned probes were inserted into either the liquid or headspace of the *Mc. capsulatus*
324 (Bath) culture medium through the Suba-Seals. The probes were removed from the
325 Suba-Seals after different incubation time (4 and 20 h, respectively), rinsed with HPLC grade
326 water, dried with lint-free tissue, and then placed into the thermal desorption tubes (Markes
327 International, UK).

328 Samples analyses were performed on a combined thermal desorption GC–MS system. The
329 volatiles were desorbed at 250°C and concentrated on a thermal desorber (Unity®, Markes
330 International Limited) at -10°C cold trap for 5 min (helium flow 50 mL min⁻¹) and then were
331 transferred onto the GC/MS system (7890A-GC with 5975C-MS, Agilent Technologies)
332 equipped with a capillary column (Agilent J&W HP- MS GC Column, 30 m, 0.25 mm, 0.25

333 μm). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1} , injector temperature,
334 250°C , and the chromatogram was obtained using the following temperature programme: 35°
335 C for 1 min; $10^\circ\text{C min}^{-1}$ to 250°C ; and then held at 250°C for 1 min. The National Institute of
336 Standards and Technology (NIST) MS search program (version 2011) was used to identify the
337 compounds based on their MS spectrum.

338 **Extraction of selenium nanoparticles produced by *Mc. capsulatus* (Bath)**

339 Freshly grown cultures (at OD_{600} of 0.5-0.8) were supplemented with $20 \text{ mg L}^{-1} \text{ SeO}_3^{2-}$ and
340 incubation was continued at 45°C with shaking in the presence of methane. After 48h the
341 development of the reddish colour had occurred, the cultures were pelleted by centrifugation
342 (at $12,500 \times g$; 10 min). SeNPs were extracted by a modification of the method published by
343 Sonkusre et al. (2014), as follows. The resultant pellet was washed and re-suspended in 10
344 mL of sterile water followed by addition of lysozyme to give a final concentration of $500 \mu\text{g}$
345 mL^{-1} , and the tube was incubated at 37°C for 3 h. The suspension was passed through a
346 French pressure cell (1500 psi, 4°C). The resultant slurry containing both cell debris and NPs
347 was washed four times at $15,000 \times g$ for 10 min with 1.5 M Tris-HCl (pH 8.3) containing 1%
348 sodium dodecyl sulfate (SDS). The resultant pellet containing SeNPs and the insoluble cell
349 wall fraction was washed and resuspended in 4 mL sterile water in a 15 mL Falcon tube, and 2
350 mL of 1-octanol were added. The solution was mixed vigorously on a vortex mixture for five
351 min and centrifuged at $2000 \times g$ for 5 min at 4°C . The tubes were then kept undisturbed at 4°
352 C for 24 hours. The upper phase and interface containing the insoluble cell fraction were
353 removed, and the bottom water phase containing SeNPs was transferred to a clean 15 mL
354 centrifuge tube. This was washed sequentially with chloroform, absolute ethanol, 70%
355 ethanol, and water at $16000 \times g$. Collected NPs were re-suspended in water and stored at 4°C .

356 **Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX)** 357 **spectrometry/high-angle annular dark-field (HAADF) scanning TEM (STEM) analysis**

358 Samples of selenite amended culture were treated and analysed as previously described
359 (Eswayah et al, 2017). The samples were examined in an FEI Tecnai F20 field emission gun
360 (FEG)-TEM operating at 200 kV and fitted with a Gatan Orius SC600A CCD camera, an
361 Oxford Instruments XMax SDD EDX detector and a high-angle annular dark-field (HAADF)
362 scanning TEM (STEM) detector.

363 For thin section analysis, after the ethanol dehydration steps, the cells were embedded in EM
364 bed 812 epoxy resin and cut into thin sections (90 nm, using a diamond knife on a Reichert
365 Ultracut S ultramicrotome). The sections were supported on copper grids and coated with

366 carbon. TEM specimen holders were cleaned by plasma prior to TEM analysis to minimize
367 contamination. Samples were examined with a high-resolution Philips CM 200 transmission
368 electron microscope at an acceleration voltage of 200 kV under standard operating conditions
369 with the liquid nitrogen anti-contaminator in place.

370 **X-ray absorption spectroscopy**

371 The conditions for the X-ray absorption spectroscopy measurements were as described
372 previously (Eswayah et al, 2017).

373 **X-ray photoelectron spectroscopy (XPS) analysis**

374 Harvested SeNPs samples were deposited on silicon wafer, left to dehydrate in the load lock of
375 the XPS instrument overnight. The analyses were carried out using a Kratos Axis Ultra DLD
376 instrument with the monochromated aluminium source. Survey scans were collected
377 between 1200 to 0 eV binding energy, at 160 eV pass energy and 1 eV intervals. High-
378 resolution C 1s, N 1s, O 1s, Se 3d and S 2p spectra were collected over an appropriate energy
379 range at 20 eV pass energy and 0.1 eV intervals. The analysis area was 700 μm by 300 μm .

380 Two areas were analysed for each sample, collecting the data in duplicate. Charge
381 neutralisation was used with intention of preventing excessive charging of the samples during
382 analysis. The data collected were calibrated in intensity using a transmission function
383 characteristic of the instrument (determined using software from NPL) to make the values
384 instrument independent. The data can then be quantified using theoretical Schofield relative
385 sensitivity factors. The data were calibrated for binding energy using the main carbon C
386 1s at 285.0 as the reference peak, and correcting all data for each sample analysis accordingly.

387 **Raman spectroscopy analysis of SeNPs**

388 Aliquots of 2 μL of SeNPs suspended in water were transferred onto a calcium fluoride (CaF_2)
389 slide and air-dried prior to Raman analysis. Raman spectra were obtained using a Horiba
390 LabRam HR and a modified Horiba LabRam HR (Wellsens Biotech. Ltd., China). Three
391 factors have been modified in this new Raman system to improve Raman spectral quality.
392 These comprise shortening the Raman light path, employing a low noise and sensitive EMCCD
393 for the Raman signal detection, and increasing incident laser power. The old and new modified
394 systems are identical except these three factors. The Raman signals were collected by a
395 Newton EMCCD (DU970N-BV, Andor, UK) utilizing a 1600×200 array of 16 μm pixels
396 with thermoelectric cooling down to -70°C for negligible dark current. A 532 nm Nd:YAG
397 laser (Ventus, Laser Quantum Ltd., UK) was used as the light source for Raman measurement.
398 A $100\times$ magnifying dry objective (NA = 0.90, Olympus, UK) was used for sample observation
399 and Raman signal acquisition. A 600 line/mm grating was used for

400 the measurements, resulting in a spectral resolution of $\sim 1 \text{ cm}^{-1}$ with 15 81 data points. The
401 laser power on sample was measured by a laser power meter (Coherent Ltd.). The Raman
402 spectra were processed by background subtraction (using spectra from cell free region on the
403 same slide) and normalization using the Labspec5 software (HORIBA Jobin Yvon Ltd., UK).

404 **Fourier transformation infrared (FT-IR) spectroscopy measurements of SeNPs**

405 In order to determine the functional groups present on the SeNPs, the FTIR spectra of SeNPs
406 were recorded on a PerkinElmer Spectrum 100 FT-IR Spectrometer equipped with an
407 attenuated total reflectance (ATR) attachment. Spectra were recorded from 4,000 to 650 cm^{-1} ,
408 and 4 scans were averaged at a resolution of 4 cm^{-1} . Extracted SeNPs were freeze dried
409 overnight and analyzed without further treatment. For comparison, the FTIR spectra of
410 samples of bacterial cells (as control) and chemically synthesized SeNPs (Chem-SeNPs) were
411 also recorded. For the controls, freshly grown cultures ($\text{OD}_{600} \sim 0.7$) of *Mc. capsulatus* (Bath)
412 were centrifuged at $11000 \times g$ for 10 min to obtain the cell pellets. The pellets were washed
413 twice with phosphate buffered saline (Sodium chloride, 150 mM, and sodium phosphate, 150
414 mM) pH 7.2, and then freeze dried overnight. The synthesis of Chem-SeNPs was done
415 according to the procedure of (Lampis *et al.*, 2017) as follows: 1.0 mL of 50 mM L-cysteine
416 (Sigma-Aldrich, Dorset, UK) solution was added dropwise into 1.0 mL of 0.1 M Na_2SeO_3 .
417 The mixed solution was then stirred for 30 min at room temperature. The Chem-SeNPs were
418 pelleted by centrifugation (at $15000 \times g$; 10 min), and then freeze dried overnight.
419

420 **Acknowledgement**

421 ASE is grateful for the award of a PhD scholarship from the Government of Libya.

422 **Conflict of Interest**

423 The authors declare no conflict of interest.

424 **Originality-Significance Statement**

425 We certify that all of the research and the conclusions are original and have not been
426 presented elsewhere.

429

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- 583

List of Figures

Figure 1 TEM thin-section micrographs of *Mc. capsulatus* (a) exposed to 20 mg L⁻¹ SeO₃²⁻, showing the extracellular locations of the Se⁰ nanospheres, HAADF-STEM imaging, showing Se nanospheres associated with the cells with EDX maps (generated from spectra collected from the indicated areas) of Se and S(b) and TEM of *Mc. capsulatus* cultures exposed to 20 mg L⁻¹ SeO₃²⁻ (c) with EDX analysis within the electron dense regions (Se⁰ nanospheres). Cells were fixed with 3% glutaraldehyde and 2% OsO₄ immediately before the analysis.

Figure 2 The FTIR spectra of freeze dried Bio-SeNPs (blue) and bacterial biomass (red) of *Mc. capsulatus* exposed to 20 mg L⁻¹ SeO₃²⁻ and harvested at OD₆₀₀ ~ 0.7, separated by centrifugation, washed with phosphate buffered saline pH 7.2 and freeze dried; as well as Chem-SeNPs (black) obtained through reaction of Na₂SeO₃ with L-cysteine. The spectra are representatives of 5 runs of the experiments.

Figure 3 Wide scan X-ray photoelectron spectra of the SeNPs produced by *Mc. capsulatus* (a) and high resolution spectra for Se 3d, C 1s, O 1s and N 1s are shown in b, c, d and e, respectively. The spectra are representatives of 2 runs of the experiments.

Figure 4 The Raman spectra of purified Se nanospheres from *Mc. capsulatus*

Figure 5 GC mass chromatograms of the liquid phase of the *Mc. capsulatus* (Bath) cultures amended with selenite (20 mg L⁻¹) at 4h (a) and 20h (b). The chromatograms were obtained by selecting the 80 m/z ion, and peak identification was achieved using the GC-MS library.

Figure 6 GC mass chromatograms of the headspace of the *Mc. capsulatus* (Bath) cultures amended with selenite (20 mg L⁻¹) at 4h (a) and 20h (b). The chromatograms were obtained by selecting the 80 m/z ion, and peak identification was achieved using the GC-MS library.

Scheme 1 A schematic diagram showing the reduction of selenite to methyl selenol with the subsequent formation of other selenium-containing species. The numbers donate the following: 1. reduction & methylation, 2. reduction & methylation, 3. polymerization, 4. exchange reactions.

Scheme 2 A schematic diagram showing the formation of methyl selenol, selenium particles and methylated derivatives.

Figure 1

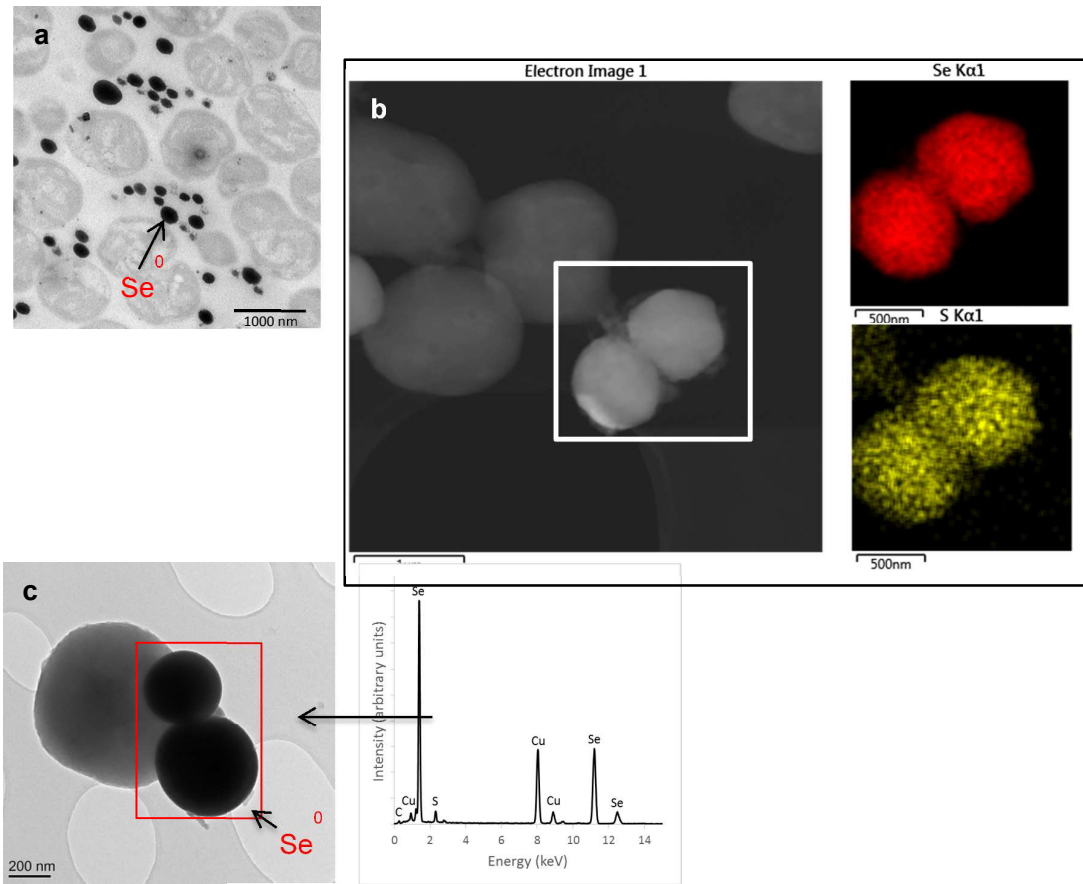
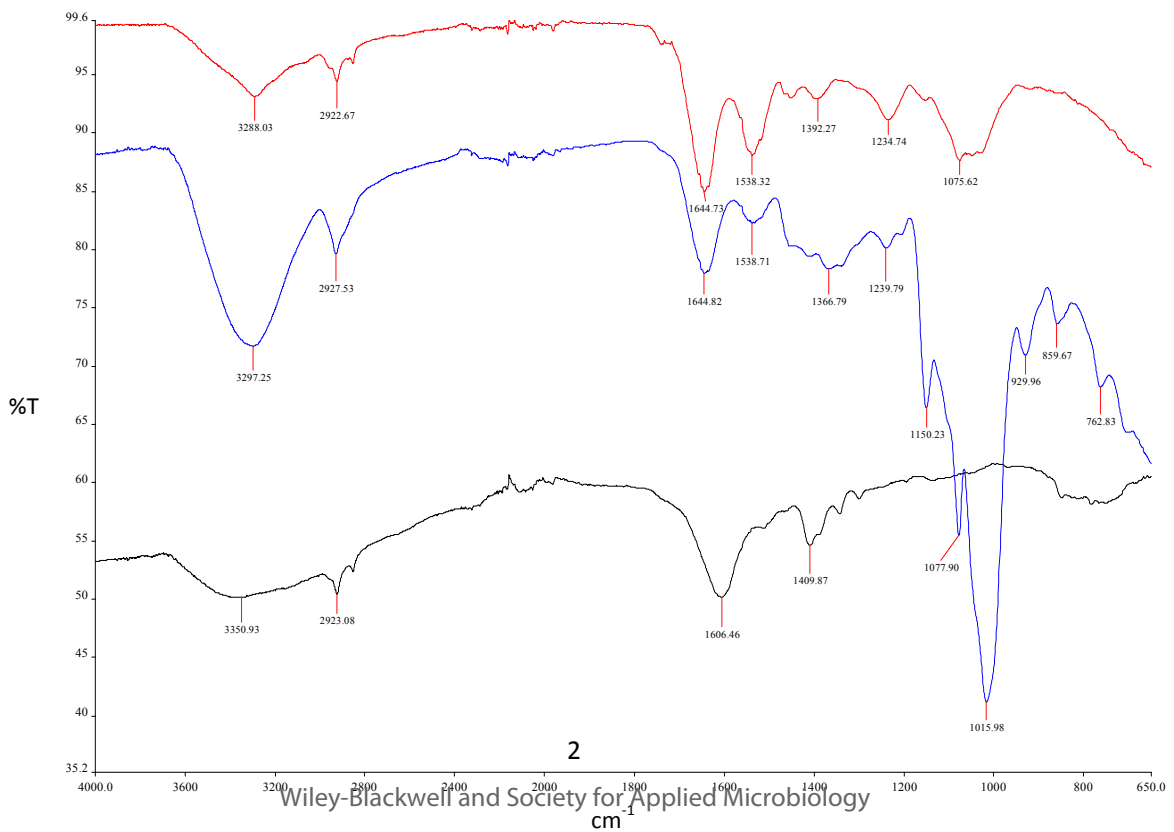


Figure 2



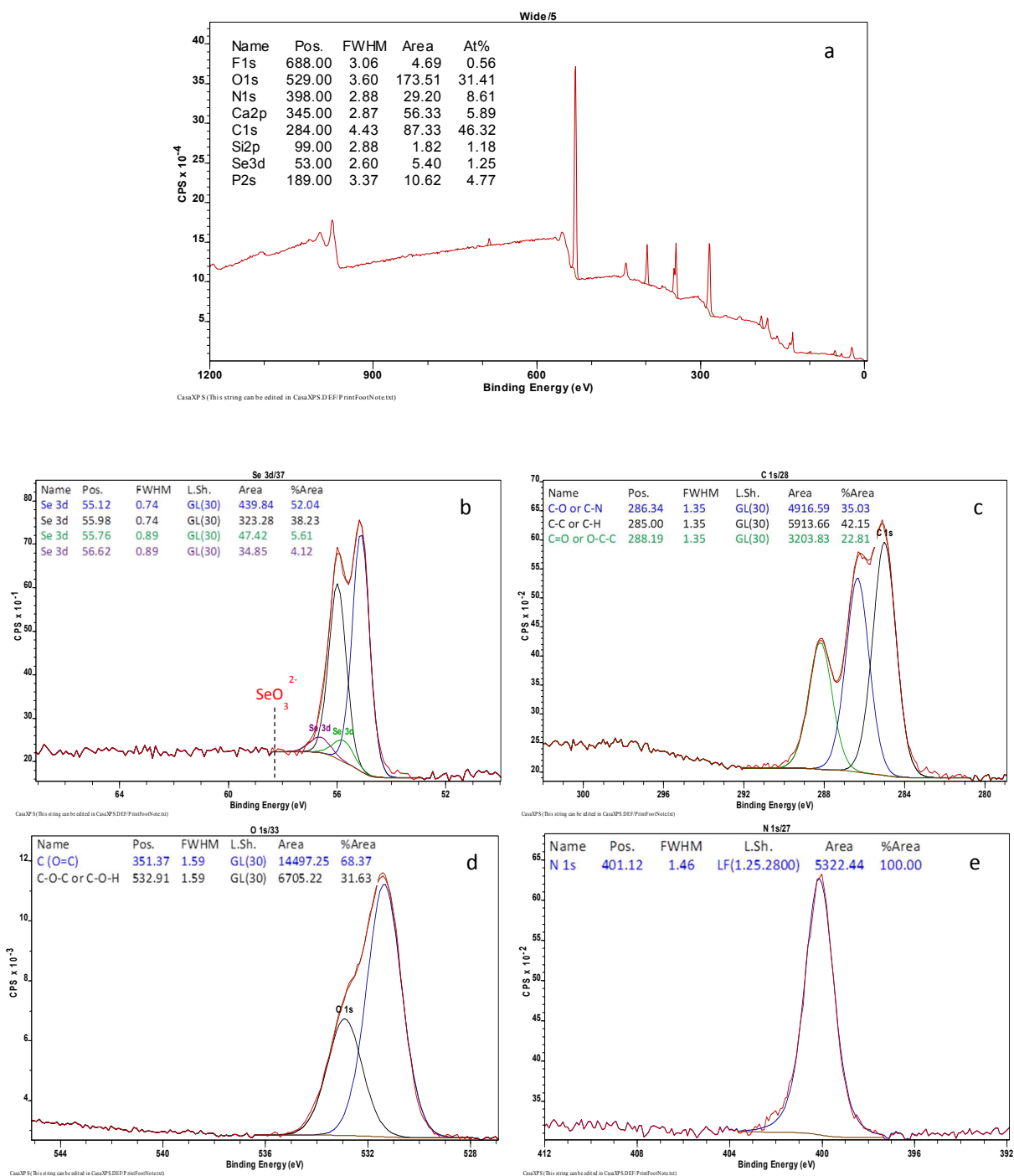
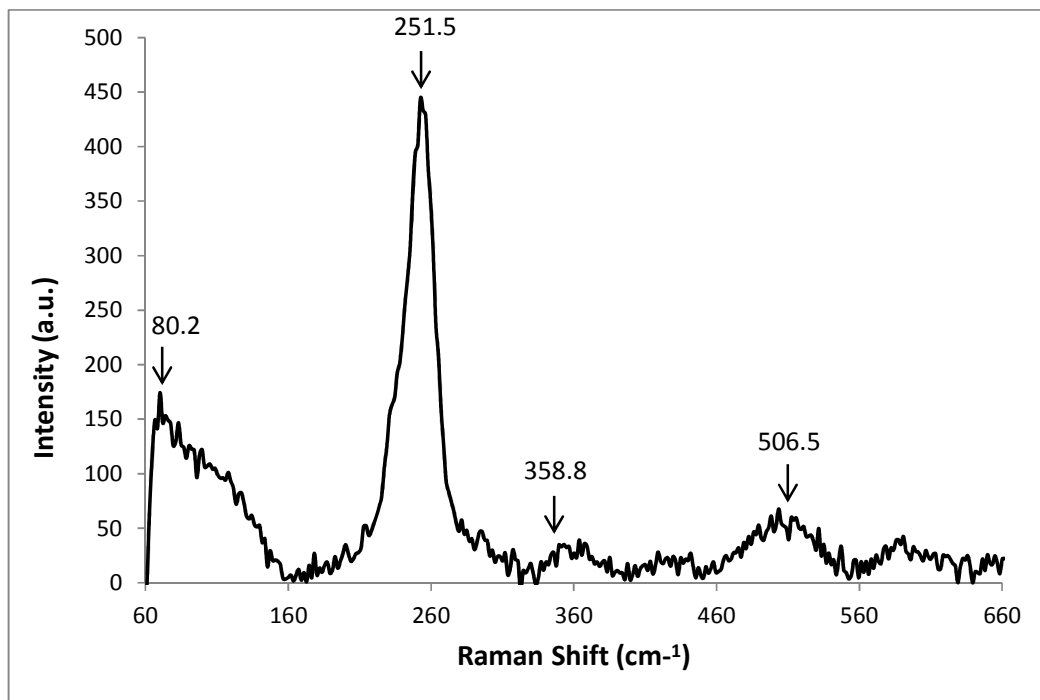
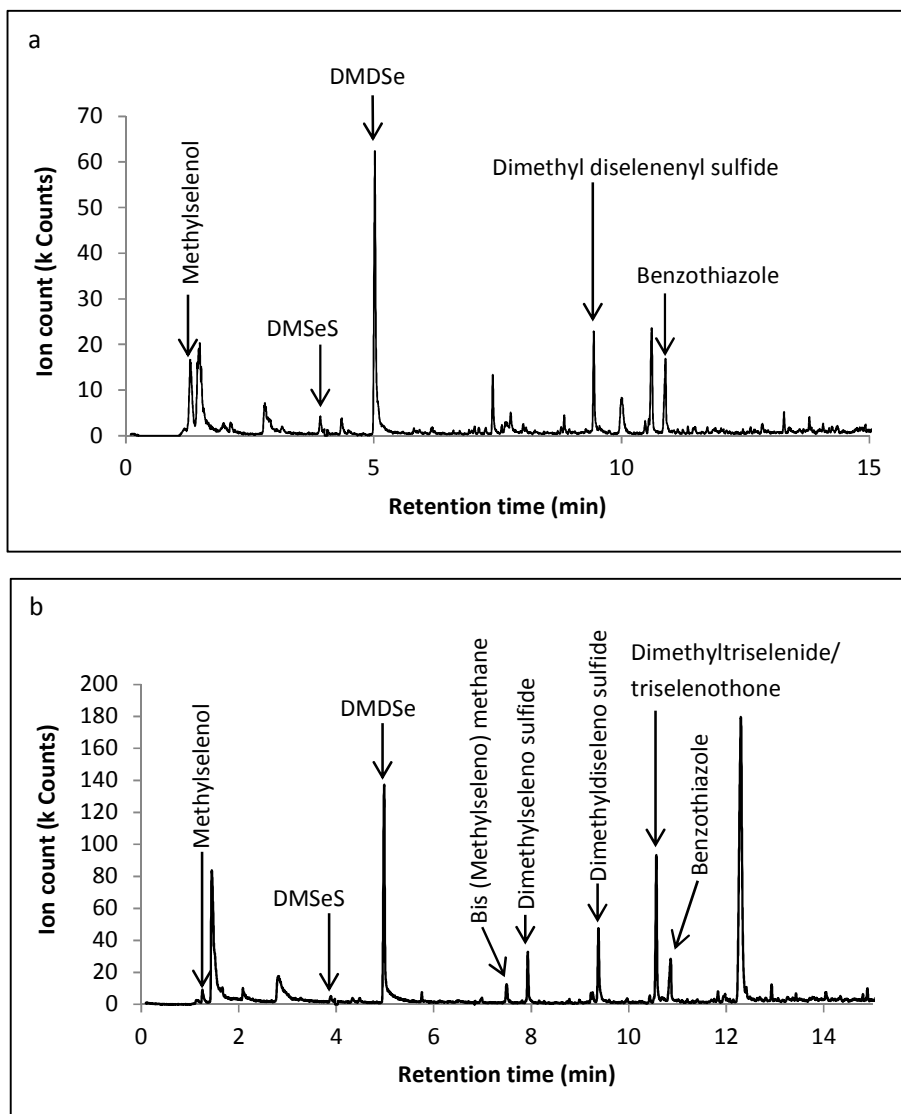


Figure 3

Figure 4



**Figure 5**

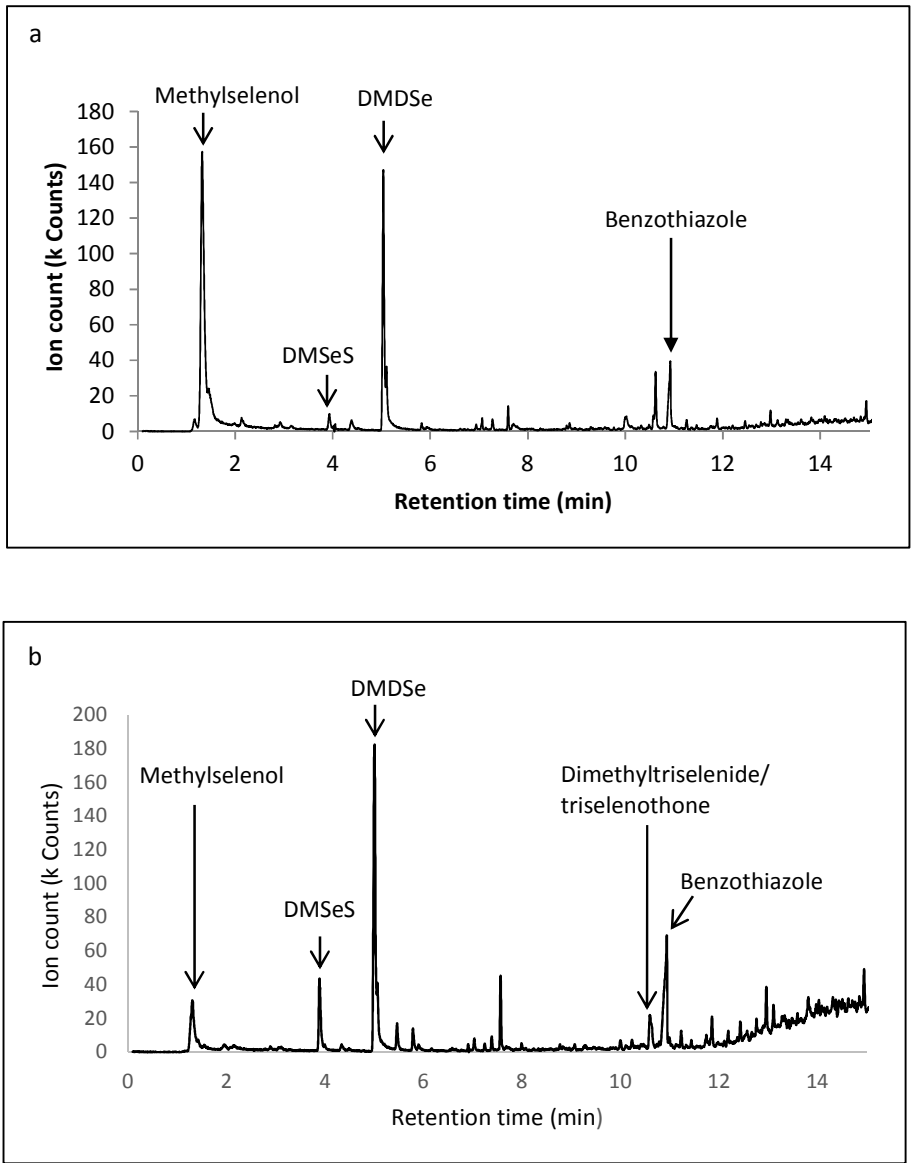
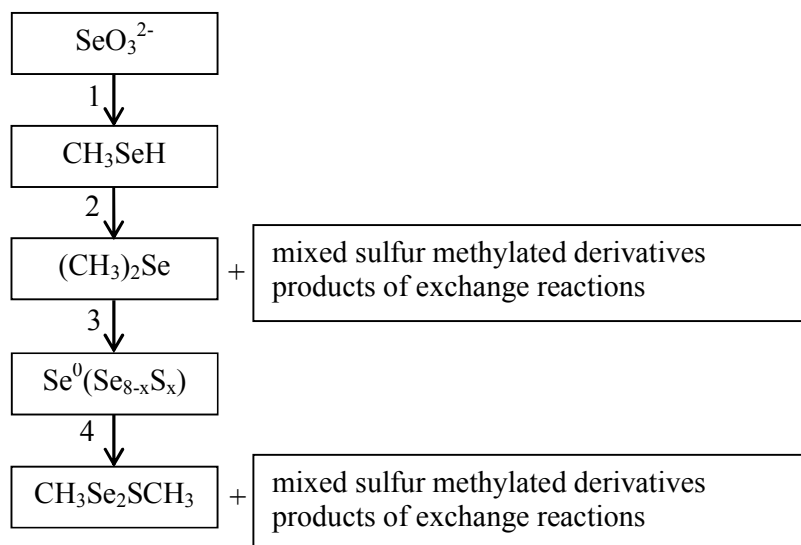
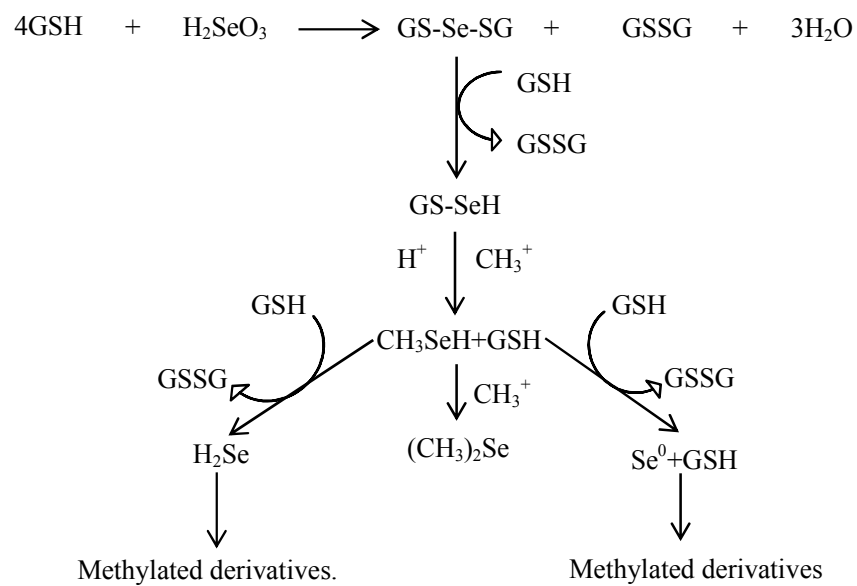


Figure 6

Scheme 1



Scheme 2



List of Tables

Table 1 Tentative assignments of main bands to the relevant functional groups (wavenumber, cm^{-1}) (Naumann *et al.*, 1995; Beekes *et al.*, 2007; Burattini *et al.*, 2008; Kamnev, 2008; Alvarez-Ordóñez *et al.*, 2011; Ojeda & Dittrich, 2012; Kamnev *et al.*, 2017).

Sample	O—H; N—H (amide A in proteins)	C—H in $>\text{CH}_2$	C=O (ester moiety)	Amide I (in proteins)	Carboxyl COO^-	Amide II (in proteins)	$-\text{CH}_2-\text{CH}_3$ (in proteins, lipids, polyesters, etc.)	C=O of COO^-	C—O—C/C—C—O (in ester moieties)	Amide III / O—P=O	C—O, C—C, C—H, C—O—C in polysaccharides, and Phosphoryl groups	"fingerprint region"
Cell biomass of <i>Mc. capsulatus</i>	3288	2922		1644		1538		1392		1234	1075	
SeNPs produced by <i>Mc. capsulatus</i>	3297	2927		1644		1538		1366		1239	1150 1077 1015	919 859 762
Chem-SeNPs		2923			1606			1409				

Table 2. A summary of the selenium- and sulfur-containing species detected in the headspace and solution after 4h and 20h incubation of *Mc. capsulatus* (Bath) in selenite amended medium using sorptive extraction in conjunction TD-GC-MS analysis

Incubation Time		Species										
		Methyl selenol	DMSeS	DMDSe	Bis (Methylseleno) methane	Dimethyl selenosulfide	Dimethyl diselenenyl sulfide	Triselenothione/Dimethyltriselenide	Benzothiazole	Diethyl sulfuxide	Propanesulfonyl	Dodecanethiol
In solution (selenite amended)	4 h	+	+	+	+	-	+	-	+	-	-	-
	20 h	+	+	+	+	+	+	+	+	+	-	-
In headspace (selenite amended)	4 h	+	+	+	-	-	-	-	+	-	-	-
	20 h	+	+	+	-	-	-	+	+	-	-	-
(Control)	20 h	-	-	-	-	-	-	-	+	-	+	+

+ = detected; - = unknown