

## **Methyl Selenol as Precursor in Selenite Reduction to Se/S Species by Methane-oxidizing Bacteria.**

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1 **Methyl Selenol as Precursor in Selenite Reduction to Se/S Species by Methane-oxidizing**  
2 **Bacteria**

3 **Methyl Selenol as Precursor in Selenite Reduction**

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17 **Abstract**

18 A wide range of microorganisms have been shown to transform selenium-containing  
19 oxyanions to reduced forms of the element, particularly selenium-containing nanoparticles.  
20 Such reactions are promising for detoxification of environmental contamination and  
21 production of valuable selenium-containing products such as nanoparticles for application in  
22 biotechnology. It has previously been shown that aerobic methane-oxidising bacteria,  
23 including *Methylococcus capsulatus* (Bath), are able to perform methane-driven conversion  
24 of selenite (SeO<sub>3</sub><sup>2-</sup>) to selenium-containing nanoparticles and methylated selenium species.

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

### 37 **Importance**

38 Aerobic methane-oxidizing bacteria are ubiquitous in the environment. Two well  
39 characterised strains, *Mc. capsulatus* (Bath) and *Methylosinus trichosporium* OB3b,  
40 representing gamma- and alpha-proteobacterial methanotrophs, can convert selenite, an  
41 environmental pollutant, to volatile selenium compounds and selenium containing  
42 particulates. Both conversions can be harnessed for bioremediation of selenium pollution  
43 using biological or fossil methane as the feedstock and these organisms could be used to  
44 produce selenium-containing particles for food, and biotechnological applications. Using an  
45 extensive suite of techniques we identified precursors of selenium nanoparticle formation,  
46 and also that these nanoparticles are made up of eight membered mixed selenium and sulfur  
47 rings.

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

50

## 51 **Introduction**

52 A key biotransformation mechanism of most microorganisms exposed to selenium oxyanions  
53 is dissimilatory reduction to nanoparticulate elemental selenium (1-4). The formation of the  
54 nanoparticles (NPs) reduces the toxicity and bioavailability of the selenium species (5-9). Not  
55 only does the formation of the NPs reduce the adverse environmental impact of the oxyanions  
56 on the microorganisms and their surroundings they also present an approach for the  
57 production of selenium NPs designed for a variety of technological, clinical, analytical and  
58 industrial applications (10-20). There is a demand for nanoparticles with new combinations  
59 of properties for specific applications. In the case of selenium-containing particles, to date  
60 these have included exploitation of their optical properties to improve the response of sensors  
61 (11), binding of heavy metal ions to effect remediation of pollution (12) and exploitation of  
62 the antitumour (16), immunomodulatory (17) and antimicrobial (19) activities of specific  
63 types of selenium-containing particles.

64 Different bacteria are known to convert selenium oxyanions into red amorphous Se NPs with  
65 distinct features. Selenium NPs produced by the selenium-respiring bacteria *Sulfurospirillum*  
66 *barnesii* and *Bacillus selenitireducens* contain predominantly  $Se_6$  chain units, while  
67 *Selenihalanaerobacter shriftii* has  $Se_8$  rings (21). *Desulfovibrio desulfuricans*, a sulfate-  
68 reducing bacterium, produces amorphous spherical submicro particles containing selenium  
69 and sulfur (22). Selenium NPs produced from selenite by *Azospirillum brasilense* are  
70 composed of cyclic  $Se_{8-x}S_x$ , with  $Se_6S_2$  the most likely structure (8). *Azospirillum thiophilum*  
71 produce amorphous selenium particles with no evidence of either Se-S or S-S bands in the

72 Raman spectrum (23). *Stenotrophomonas bentonitica* produces amorphous Se<sup>0</sup> nanospheres  
73 that subsequently transformation to one-dimensional (1D) trigonal selenium nanostructure  
74 where sulfur is associated with the SeNPs (24). In an earlier study, (25), we presented indirect  
75 evidence from transmission electron microscopy (TEM) imaging with energy dispersive X-  
76 ray spectrometry (EDX) measurements to show that sulfur was associated with amorphous  
77 selenium in the extracellular NPs that are produced in a methane-dependent fashion from the  
78 reduction of selenite by the aerobic obligate methane-oxidizing bacteria *Methylococcus*  
79 *capsulatus* (Bath) and *Methylosinus trichosporium* OB3b.

80 Besides the above-mentioned studies on the biotransformation of selenium oxyanions most  
81 investigations have focused on the formation of the NPs. A few others have identified  
82 concomitant release of volatile selenium species into the headspace gas (26, 27). However,  
83 none of these approaches has provided enough information to enable the elucidation of the  
84 processes leading to the formation of the amorphous NPs. Indeed, the formation of the  
85 extracellular amorphous NPs forms as reported in many studies (18, 21, 28-30) may indicate  
86 limited direct involvement of the microorganisms in their formation, although the initial  
87 reactants may have been produced in the bacteria. The size of the extracellular nanoparticles  
88 produced by *Mc. capsulatus* Bath increased with time (25), which suggested that the growth  
89 of the NPs is a result of abiotic reactions in the culture medium outside the cells. The  
90 formation of mixed chalcogenide species by exchange reactions, when both Se and S species  
91 are present in the gaseous and solution phase, has been reported (31, 32). It is probable that  
92 similar reactions occur in the culture medium solution resulting eventually to the formation of  
93 the nanoparticles. Here, it was our intention to observe the process of nanoparticle formation  
94 and structure of the nanoparticles in greater detail and to be better able to understand the  
95 biotic and abiotic transformations occurring in the methanotroph-mediated transformation of  
96 selenite. To this end, both selenium- and sulfur-containing species were sampled from the

97 headspace and solution of selenite amended and control samples at fixed times by sorptive  
98 extraction in conjunction with analysis by thermal desorption - gas chromatography- mass  
99 spectrometry (TD-GC-MS) to identify the compounds. In parallel, the formed NPs were  
100 characterised by a range of physical techniques, namely; attenuated total reflectance Fourier  
101 transformation infrared spectroscopy (ATR-FTIR), Raman spectroscopy, transmission  
102 electron microscopy (TEM) and energy dispersive X-ray (EDX) spectrometry, X-ray  
103 absorption spectroscopy (XAS), and X-ray photoelectron spectroscopy (XPS). Herein, the  
104 results obtained from these measurements are used to inform the formation and elucidation of  
105 the structure of the sulfur-doped red amorphous selenium NPs produced when a methane-  
106 oxidizing bacterium reduces selenite. *Mc. capsulatus* (Bath) was chosen for these  
107 experiments since it transforms selenite at approximately five times the rate of *Ms.*  
108 *trichosporium* OB3b (25).

## 109 **Results**

110 Previous investigations with the methanotroph species *Mc.capsulatus* (Bath) showed that the  
111 sizes of the NPs grew rapidly from an average of  $220 \pm 51$  nm in the first 4 hrs to about 400  
112  $\pm 77$  nm in the next 44 hrs as previously published (25). Here, high angle annular dark field-  
113 scanning transmission electron microscopy (HAADF-STEM) imaging and TEM thin-section  
114 micrographs of cultures producing the nanoparticles were performed and it appears that the  
115 NPs are associated extracellularly with the cells (see Fig. 1a). Furthermore, the spatial  
116 distributions of Se and S in the EDX maps overlap, indicative of a spatial and likely structural  
117 association, suggesting the formation of mixed chalcogenide nanoparticles (see Fig.1b). The  
118 intensity of the Se signals was, however, much higher than that for S (see Fig. 1c). In addition,  
119 examination of the  $S_{k\alpha}$  map (Fig. 1b) reveals that not only was there sulfur in the particles but

120 there was a “trail” of the element linking the particles to the cells, suggesting likely sulfur-  
121 containing proteins from the bacterial cells.

122 Se K-edge X-ray Absorption Near-Edge Structure (XANES) spectra of the particles formed  
123 by *Mc. capsulatus* (not shown) are similar to those of red amorphous Se with no detectable  
124 residual selenite present in the samples. Previous shell fitting of the Extended X-ray  
125 Absorption Fine-Structure (EXAFS) spectra formed by *Mc. capsulatus* (25) showed the  
126 characteristics of amorphous elemental Se<sup>0</sup>, with two atoms at a distance of 2.35Å, however  
127 this is slightly shorter than 2.36Å expected for red elemental Se (33,34). The shorter bond  
128 length is an indication that there may be partial substitution of Se for the smaller S atom in  
129 the structure of the amorphous red elemental Se. This is a feature that has been observed for  
130 mixed Se and S nanoparticles formed by *A. brasilense* (35). In order to unravel the make-up  
131 of the nanoparticles, the surfaces of the particles were characterized by FTIR, XPS and  
132 Raman spectroscopy.

### 133 **FTIR Analysis**

134 The FTIR spectra of the freeze-dried selenium nanoparticles produced by the *Mc. capsulatus*  
135 in liquid NMS medium amended with selenite, the Chem-SeNPs (in-house produced  
136 selenium nanoparticles as described in the experimental section) and the bacterial biomass are  
137 shown in Figure 2. The assignments of the bands, which are based upon the characteristic  
138 vibrational frequencies of the different types of covalently bonded atoms, are summarised in  
139 Table 1. The peak centred at 3297 cm<sup>-1</sup> corresponds to the –OH and –NH stretching  
140 vibrations of the amine and carboxylic groups. Peaks at 2927 cm<sup>-1</sup> corresponded to the  
141 aliphatic saturated C–H stretching modes (30, 36). The peaks at 1644, 1538, and 1239 cm<sup>-1</sup>  
142 are characteristic of amide I, amide II, and amide III bands of proteins, respectively (37, 38).  
143 The symmetrical stretch of carboxylate group can be attributed to the bands observed at 1366

144  $\text{cm}^{-1}$ . The peaks at 1150, 1077 and  $1015 \text{ cm}^{-1}$  corresponded to the C–O stretching vibrations  
145 of C–O–C groups (37, 39). The presence of phosphoryl groups was confirmed by the peak at  
146  $929 \text{ cm}^{-1}$ . Additionally, peaks at 859 and  $762 \text{ cm}^{-1}$  (fingerprint region) could be mainly  
147 attributed to aromatic ring vibrations of aromatic amino acids (tyrosine, tryptophan,  
148 phenylalanine) and possibly nucleotides (40, 41).

149 The FTIR spectra of SeNPs of *Mc. capsulatus* differ from those of the bacterial biomass  
150 (control) and the Chem-SeNPs. The main difference between the spectra is that the Bio-  
151 SeNPs exhibit more peaks in the protein and polysaccharide vibration region, indicating the  
152 presence of proteins and polysaccharides in the biomacromolecules capping the SeNPs (20,  
153 30, 42, 43).

154 By contrast, Chem-SeNPs obtained through reaction of  $\text{Na}_2\text{SeO}_3$  with L-cysteine displayed a  
155 broad absorption band around  $3350 \text{ cm}^{-1}$  and absorption band at  $2923 \text{ cm}^{-1}$  that are assigned  
156 to O–H vibrations of the absorbed  $\text{H}_2\text{O}$  and C–H vibration in the alkyl chain of L-Cys,  
157 respectively. The peak at  $1606 \text{ cm}^{-1}$  can be mainly attributed to C=O vibrations. It is  
158 noteworthy that the presence of organic residues such as carbohydrates, lipids, and proteins  
159 on the surface of biogenic SeNPs were completely absent in the Chem-SeNPs spectrum (see  
160 Figure 2). FTIR spectra of the Bio-SeNPs separated from the *Mc. capsulatus* cells showed  
161 bands typical of proteins, polysaccharides and lipids associated with the particles.

## 162 **XPS analysis of the particle surface**

163 The surface composition of the harvested red particles was obtained by XPS, which is a  
164 surface technique that obtains information about the elemental composition of a sample  
165 according to the emission of photoelectrons when the sample is irradiated with X-ray energy.  
166 The signals observed in XPS can be identified based on their binding energy as arising from  
167 expulsion of electrons from specific energy levels within each element on the surface (rather



168 than the interior) of the particles. Hence, the surface elemental content can be summarised  
169 from the full range XPS spectrum (Supplemental Figure 1a). In addition to the selenium  
170 which is present at a concentration of 1.25% (atomic percent), there are five other elements:  
171 carbon (46.32%), oxygen (31.41%), nitrogen (8.61%), calcium (5.89%) and phosphorus  
172 (4.77%) that were detected on the surface of the particles (see Supplemental Figure 1a and  
173 the elemental information therein). The presence of the first three elements is an indication  
174 that there are organic molecules on the particle surfaces. The high resolution spectral scans,  
175 which show the regions due, respectively, to Se, C, N, and O, and the assigned chemical  
176 species from the core level XPS spectra of C 1s, N 1s, O 1s bands are shown in Figures 3b-e.  
177 These spectra are consistent with the presence of biomolecules, including proteins, on the  
178 surface of the particles. The spectrum for 3d Se shows a doublet which does not show base-  
179 line resolution. However, the fitted deconvoluted peaks in this region (Supplemental Figure  
180 1b) show two predominant bands at 55.16 and 56.02 binding energy (eV), and two minor  
181 peaks at 55.75 and 56.61 eV, respectively, the latter pair of peaks resulting from the Se 3d  
182 peak split by spin orbit coupling into Se 3d<sub>5/2</sub> and Se 3d<sub>3/2</sub>. The key conclusion enabled by  
183 these results is that the observed range of binding energies between 55.16 and 56.61eV is  
184 indicative of the presence of reduced selenium species, including elemental selenium at the  
185 surface of the particles (44). The binding energy expected for the Se 3d electrons in selenite  
186 (SeO<sub>3</sub><sup>2-</sup>) is indicated in Supplemental Figure 1b; it is clear that no signal at this position is  
187 observed.

### 188 **Raman characterisation of the amorphous particles**

189 Vibrational spectroscopy particularly Raman spectroscopy (which is a visible light scattering  
190 technique that gives information complementary to that from IR spectroscopy) has been the  
191 technique of choice for the characterization of Se<sub>n</sub> allotropes, and aggregates. The  
192 deconvoluted Raman spectrum obtained between 50-600 cm<sup>-1</sup> shows the shift in frequency of

193 the scattered light that corresponds to the vibrational spectrum of the harvested SeNPs  
194 (Figure 3). There are four bands which are visible: the main band at  $251.5\text{ cm}^{-1}$  and smaller  
195 ones at  $80.2$ ,  $358.8$  and  $506.5\text{ cm}^{-1}$ , respectively. All of the bands were present in all of the  
196 scans of samples collected at different time points: 6, 24 (supplemental Figure 2) and 48 h  
197 (Figure 3). The band at  $506.5\text{ cm}^{-1}$  was more prominent than that at  $358.8\text{ cm}^{-1}$ . The band at  
198  $80.2\text{ cm}^{-1}$ , which is a shoulder, is only visible in the deconvoluted spectrum.

199 It has been proposed that amorphous selenium is composed of a mixture of  $\text{Se}_n$  rings and  
200 helicoidal chains (45-47). The proportion of each depends on chemical and physical  
201 conditions under which the samples are made, and the treatments to which they have been  
202 subjected. According to Carini et al the band at around  $250\text{ cm}^{-1}$  is characteristic of  
203 amorphous selenium (48). The symmetrical band at  $251.5\text{ cm}^{-1}$ , with full width half maxima  
204 of  $30\text{ cm}^{-1}$  found in this study, is characteristic of Se-Se stretching vibration in pure  $\text{Se}_8$  (45,  
205 49, 50) with its deformation vibration at  $80.2\text{ cm}^{-1}$ . However, in the study of mixed selenium  
206 and sulfur alloys by Machado et al, the authors state that as the sulfur concentration increases  
207 in mixed amorphous selenium, the peak at  $234\text{ cm}^{-1}$  usually associated with Se chains  
208 decreases in intensity and the band at  $250\text{ cm}^{-1}$ , usually associated with selenium rings  
209 increases in intensity. They also observed the appearance of a band at  $352\text{ cm}^{-1}$  as the  
210 selenium to sulfur ratio increases to either 4:1 or 7:3 (51). The band at  $358.8\text{ cm}^{-1}$  has been  
211 assigned to S-Se stretching vibration (52, 53). The band at  $506.5\text{ cm}^{-1}$  is probably due to the  
212 Se-Se overtone band of the fundamental band at  $251.5\text{ cm}^{-1}$ . The S-S vibration band is not  
213 seen when the S composition is below that found in  $\text{Se}_6\text{S}_2$  (45). Therefore it is probable that  
214 the composition of the harvested particles is  $\text{Se}_{8-x}\text{S}_x$ , where  $x$  is equal to or greater than 2.

215

216

## 217 **Speciation of selenium and sulfur in the medium solution and headspace**

218 The GC-MS chromatograms of the species found in both the headspace and solution of the  
219 selenite amended medium at 4h and 20h are shown in Supplemental Figure 3. The earlier  
220 time was chosen because the formation of the particles and therefore the red colour of the  
221 solution were barely discernible. A summary of the selenium- and sulfur-containing species  
222 is given in Table 2. Examination of the data in Table 2 showed that after 4 h, three  
223 compounds: methyl selenol ( $\text{CH}_3\text{SeH}$ ), dimethyl selenenyl sulfide ( $\text{DMSeS}$ ;  $\text{CH}_3\text{-Se-S-CH}_3$ )  
224 and dimethyl diselenide ( $\text{DMDSe}$ ;  $\text{CH}_3\text{-Se-Se-CH}_3$ ) were detected in both the solution and  
225 headspace. In addition dimethyl diselenenyl sulfide ( $\text{CH}_3\text{-Se}_2\text{S-CH}_3$ ) and Bis(methylseleno)  
226 methane ( $\text{CH}_3\text{-Se-CH}_2\text{-Se-CH}_3$ ) were also found in the solution. At 20 h, dimethyl selenenyl  
227 disulfide ( $\text{CH}_3\text{-SeS}_2\text{-CH}_3$ ) was detected in the solution in addition to triselenothone/  
228 dimethyltriselenide ( $\text{CH}_3\text{-Se-Se-Se-CH}_3$ ) which was detected in both the solution and  
229 headspace. The control in the absence of selenite showed no selenium-containing species,  
230 though three sulfur-containing species were observed (Table 2).

## 231 **Discussion**

232 The results from the previous kinetic experiments showed that there were increases in  
233 particles sizes with incubation time (25) leading us to hypothesize that much of the structure  
234 of the particles was formed in the extracellular space. If data support this hypothesis, then the  
235 key reactions resulting in the increase in the particle sizes are essentially abiotic in nature,  
236 though they require electrons ultimately derived from methane. Consequently, the clues to the  
237 structural formation of the particles must lie in the nature and identity of the compounds that  
238 are concomitant in the solution and headspace of the nascent particles. It was therefore  
239 essential to sample for selenium- and sulfur-containing compounds in both the headspace and  
240 solution, followed by their analyses and identification.

241 The FTIR spectra of the SeNPs produced by *Mc. capsulatus*, samples of biomass of the strain  
242 (control), as well as the Chem-SeNPs were recorded in order to identify the functional groups  
243 capping the synthesized SeNPs. These results were consistent with the particles being coated  
244 with cell-derived proteins and other biomolecules and are in line with their TEM images  
245 showing a thin layer over the particles, in addition to strong carboxylate bands, which may  
246 stabilize the SeNPs structure and morphology.

247 The XPS results show two Se containing species, and other organic constituents. In assigning  
248 the Se bands, it is essential to link the structure of the particles to the selenium-containing  
249 species that have been identified in the solution (see the discussion of the TD-GC-MS results  
250 below). Since data for the exact compounds are not available, structures that may be similar  
251 to these in the particles have been selected. The major band at 55.16 eV, has been assigned to  
252 the compound:  $(\text{CH}_3)_2\text{NC}(\text{Se})\text{SeC}(\text{Se})\text{N}(\text{CH}_3)_2$  (54). This is a reasonable fit to the results  
253 obtained in this study not only because of the presence of selenium but also the content of the  
254 methyl groups and nitrogen. The nitrogen could account for the single band at 400.10 eV  
255 usually assigned to amine nitrogen. The bands at 56.02 eV has been assigned to  $(-\text{CSeC}(\text{CH}_3)\text{C}(\text{CH}_3)\text{Se}-)_2$  (55). Both of these assignments are an indication of the presence of  
256 long chain of selenium-containing methylated species. Missing from both spectra is oxygen  
257 which is presumably present as C=O, and either C-O-C or C-O-H at 531.58 and 532.84 eV,  
258 respectively. However, the presence of Se-C was not detected in the spectrum thus indicating  
259 the amount of carbon directly bound to selenium was low. Furthermore, because of the high  
260 concentration of selenium in relation to sulfur, the signal for the latter could not be resolved  
261 and identified.

263 Whereas, both FTIR and XPS provided information on the forms in which the elements are  
264 present on the particle surface, Raman spectroscopy enables the identification of the basic  
265 structural make-up of the particles. The intensity of the band  $251.5\text{ cm}^{-1}$  is indicative of the

266 predominance of the Se-Se bonds in the structure of the particles. It is probable that S is  
267 integral to the mixed particle structure  $Se_{8-x}S_x$ , and not as an  $S_8$  impurity in the particles. The  
268 low available sulfur content in the medium makes the formation of an S-S homonuclear bond  
269 in the mixed particle structure highly unlikely (52).

270 We have previously reported that methyl selenol is produced and detected in the head space  
271 only when selenite is in the starting medium (25). Methylated selenium-containing volatiles  
272 are known to be produced during transformation of selenium-containing species by other  
273 types of bacteria (reviewed in [5]), but the presence of methyl selenol has not been reported  
274 and may be unique to methanotrophs. Besides the presence of methyl selenol in the  
275 headspace, methyl selenoacetate was also detected. However, in the present study methyl  
276 selenoacetate was not detected when the headspace sorptive extraction probes were deployed  
277 for sample collection. No methyl selenol was detected when either the harvested or  
278 chemically synthesized nanoparticles were added to the medium in the absence of selenite.  
279 Therefore, we propose that methyl selenol may be the precursor of all of the methylated  
280 selenium species as well as the selenium-containing nanoparticles. If this is the case, the first  
281 step in the biotransformation of selenite by *Mc. capsulatus* would involve the reduction and  
282 methylation of selenite to methyl selenol followed by the formation of the other selenium-  
283 and sulfur-containing species. Indeed, the formation of some of the latter species requires the  
284 presence of the nascent selenium particles.

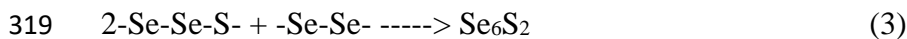
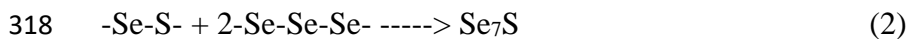
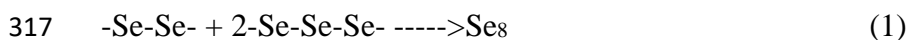
285 Based on these observations a possible pathway for the formation of the particles and the  
286 other products of biotransformation of selenite can be outlined as in Figure 4. A series of  
287 reactions proposed by Ganther (56, 57) and outlined by Xu and Barton (22) implicate  
288 glutathione (GSH) and GSH reductase in the production of elemental selenium. This is  
289 possible in *Mc. capsulatus*, in which glutathione is known (58), though would not account for  
290 possible differences in pathway from other microorganisms in which GSH and GSH

291 reductase are widespread. The proposed steps leading up to the formation of methyl selenol  
292 involving GSH is shown in Figure 5. Whilst the transformation of selenite by whole cells in  
293 culture requires the presence of the growth substrate methane, previous work showed that  
294 selenite was transformed by concentrated cell fractions (cell walls, membranes and the  
295 soluble fraction) to the red particulate material in the absence of methane. The fact that the  
296 greatest activity was found in the cell wall-enriched fraction (25) is consistent with the  
297 extracellular growth of the particles.

298 The presence of the sulfur-containing benzothiazole was detected, whether or not the culture  
299 had been amended with selenite. The sulfur-containing volatile species dodecanethiol and  
300 propanesulfonyl were detected only in the control cultures without selenite (Table 2). Such  
301 sulfur-containing species may be the source of sulfur in the structure of the selenium-rich  
302 particles. Evidence of the formation of the longer chains of the selenium- containing species  
303 can be seen in the nature of the compounds that are found in the solution after hours of  
304 incubation, first after 4h, dimethyl diselenenyl sulfide was detected, and subsequently  
305 dimethylselenodisulfide, dimethyltriselenide and bis(methylseleno) methane were detected  
306 after 20h. More complex mixed Se and S compounds are formed in the medium with time.  
307 The formation of the mixed chalcogenides of Se and S is hardly surprising since S may be  
308 available from the reduction of sulfate in the growth medium. Genes for assimilatory uptake  
309 and reduction of sulfate are present within the *Mc. capsulatus* genome (59) and the strain  
310 grows using sulfate as sole sulfur source. A key question therefore is how the solution  
311 chemistry relates to the observed structural features of the Se particulates.

312 It is likely that these longer chains polymerize to form  $Se_x$  or  $Se_{8-x}S_x$  linear or cyclic  
313 structures (32). Indeed, all chalogen elements have the tendency to form cyclic allotropes  
314 (60). The dominant allotrope will depend on the experimental conditions. Examples of

315 exchange reactions that may occur based on the presence of the detected selenium- and  
316 sulfur- containing species are shown in the following equations:



320 As can be seen a variety of nanocomposites can be formed. It is noteworthy that these  
321 structures are methylated providing an organic coating associated with the nanoparticles (61)  
322 Similar exchange reactions have been shown to occur when mixed Se and S complexes are  
323 present in the same solution (32). Indeed these reactions are known to occur in amorphous  
324 selenium semiconductors (62). The Raman results indicate these are not open chain clusters  
325 but cyclic structures with the  $Se_8$  structure dominant and the probable presence of small  
326 amounts of Se-S bonds. The source of sulfur must be from the reduction of sulfate used in the  
327 growth medium since this is the only sulfur added to the system. If it were desired to reduce  
328 the sulfur content of the particles, it may be possible to achieve this by reducing the amount  
329 of sulfate in the culture medium so that sulfur becomes growth-limiting. Since it may be that  
330 the cells can produce the nanoparticles after they have ceased to grow, this may allow the  
331 selenite to be added to the medium after the sulfate is used up, so that less sulfur would be  
332 available for incorporation into the particles.

333 In this study, we demonstrate for the first time that in the reduction of selenite by *Mc.*  
334 *capsulatus* (Bath), a methane oxidizing bacterium, methyl selenol is the likely precursor for  
335 the formation of methylated selenium-containing and mixed chalcogenides species. This  
336 reaction may be biotechnologically useful for bioremediation of selenite and production of  
337 selenium-sulfur nanoparticles for electronics and other applications, using cheaply available

338 methane as the feedstock. If this reaction is widespread among methanotrophs, it may also  
339 have significance in selenium transformations in the environment. To date, it has been shown  
340 that four isolated strains, including methanotrophs of the gammaproteobacteria (*Mc.*  
341 *capsulatus* Bath, *Mm. buryatense* and *Mm. alkaliphilum*) and alphaproteobacteria (*Ms.*  
342 *trichosporium* OB3b) perform this reaction.

343 Subsequent exchange reactions between the species result in the formation of the amorphous  
344 allotropic form of selenium, cyclic  $Se_8$  with sulfur in its structure. The nature of the molecular  
345 mediators in reduction of selenite, supplying sulfur that is integral to the structure of the  
346 nanoparticles and supplying the methyl groups found in the volatile selenium containing  
347 products remain to be identified.

## 348 **Materials and Methods**

### 349 **Bacterial strains and growth conditions**

350 The methanotrophic bacterium *Methylococcus capsulatus* (Bath) (NCIBM 11132) was grown  
351 and propagated aerobically using methane as the carbon and energy source as previously  
352 described (25). For these experiments the initial selenite concentration used was 20 mg L<sup>-1</sup>.

### 353 **Detection of solution and volatile selenium species**

354 Solution and volatile selenium-containing species were sampled by immersive sorptive  
355 extraction using sampling probes (HiSorb probe, Markes International, UK) from either the  
356 solution or headspace. Extension screw-on arms were fabricated for each probe so that they  
357 could be inserted through the Suba-Seals used to seal the necks of the culture flasks. To  
358 ensure that the probes and tubes were contamination free, before use, the probes and tubes  
359 were preconditioned with helium at flow rate of 90 mL min<sup>-1</sup> using the following temperature  
360 programme: 15 min at 100 °C, 15 min at 200 °C, 15 min at 300 °C and 15 min at 335 °C.



361 The preconditioned probes were inserted into either the liquid or headspace of the *Mc.*  
362 *capsulatus* (Bath) culture medium through the Suba-Seals. The probes were removed from  
363 the Suba-Seals after different incubation time (4 and 20 h, respectively), rinsed with HPLC  
364 grade water, dried with lint-free tissue, and then placed into the thermal desorption tubes  
365 (Markes International, UK).

366 Samples analyses were performed on a combined thermal desorption GC–MS system. The  
367 volatiles were desorbed at 250°C and concentrated on a thermal desorber (Unity®, Markes  
368 International Limited) at -10°C cold trap for 5 min (helium flow 50 mL min<sup>-1</sup>) and then were  
369 transferred onto the GC/MS system (7890A-GC with 5975C-MS, Agilent Technologies)  
370 equipped with a capillary column (Agilent J&W HP-5MS GC Column, 30 m, 0.25 mm, 0.25  
371 µm). Helium was used as the carrier gas at a flow rate of 1 mL min<sup>-1</sup>, injector temperature,  
372 250°C, and the chromatogram was obtained using the following temperature programme:  
373 35°C for 1 min; 10°C min<sup>-1</sup> to 250°C; and then held at 250°C for 1 min. The National  
374 Institute of Standards and Technology (NIST) MS search program (version 2011) was used to  
375 identify the compounds based on their MS spectrum.

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

377 Freshly grown cultures (at OD<sub>600</sub> of 0.5-0.8) were supplemented with 20 mg L<sup>-1</sup> SeO<sub>3</sub><sup>2-</sup> and  
378 incubation was continued at 45°C with shaking in the presence of methane. After 48h the  
379 development of the reddish colour had occurred, the cultures were pelleted by centrifugation  
380 (at 12,500 × g; 10 min). SeNPs were extracted by a modification of the method published by  
381 Sonkusre et al. (63) , as follows. The resultant pellet was washed and re-suspended in 10 mL  
382 of sterile water followed by addition of lysozyme to give a final concentration of 500 µg mL<sup>-1</sup>  
383 and the tube was incubated at 37°C for 3 h. The suspension was passed through a French  
384 pressure cell (1500 psi, 4°C). The resultant slurry containing both cell debris and NPs was  
385 washed four times at 15,000 × g for 10 min with 1.5 M Tris-HCl (pH 8.3) containing 1%

386 sodium dodecyl sulfate (SDS). The resultant pellet containing SeNPs and the insoluble cell  
387 wall fraction was washed and resuspended in 4 mL sterile water in a 15 mL Falcon tube, and  
388 2 mL of 1-octanol were added. The solution was mixed vigorously on a vortex mixture for  
389 five min and centrifuged at  $2000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The tubes were then kept undisturbed  
390 at  $4^{\circ}\text{C}$  for 24 hours. The upper phase and interface containing the insoluble cell fraction were  
391 removed, and the bottom water phase containing SeNPs was transferred to a clean 15 mL  
392 centrifuge tube. This was washed sequentially with chloroform, absolute ethanol, 70%  
393 ethanol, and water at  $16000 \times g$ . Collected NPs were re-suspended in water and stored at  $4^{\circ}\text{C}$ .

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

396 Samples of selenite amended culture were treated and analysed as previously described (25).  
397 The samples were examined in an FEI Tecnai F20 field emission gun (FEG)-TEM operating  
398 at 200 kV and fitted with a Gatan Orius SC600A CCD camera, an Oxford Instruments XMax  
399 SDD EDX detector and a high-angle annular dark-field (HAADF) scanning TEM (STEM)  
400 detector.

401 For thin section analysis, after the ethanol dehydration steps, the cells were embedded in EM  
402 bed 812 epoxy resin and cut into thin sections (90 nm, using a diamond knife on a Reichert  
403 Ultracut S ultramicrotome). The sections were supported on copper grids and coated with  
404 carbon. TEM specimen holders were cleaned by plasma prior to TEM analysis to minimize  
405 contamination. Samples were examined with a high-resolution Philips CM 200 transmission  
406 electron microscope at an acceleration voltage of 200 kV under standard operating conditions  
407 with the liquid nitrogen anti-contaminator in place.

408 **X-ray absorption spectroscopy**

409 The conditions for the X-ray absorption spectroscopy measurements were as described  
410 previously (25).

#### 411 **X-ray photoelectron spectroscopy (XPS) analysis**

412 Harvested SeNPs samples were deposited on silicon wafer, left to dehydrate in the load lock  
413 of the XPS instrument overnight. The analyses were carried out using a Kratos Axis Ultra  
414 DLD instrument with the monochromated aluminium source. Survey scans were collected  
415 between 1200 to 0 eV binding energy, at 160 eV pass energy and 1 eV intervals. High-  
416 resolution C 1s, N 1s, O 1s, Se 3d and S 2p spectra were collected over an appropriate energy  
417 range at 20 eV pass energy and 0.1 eV intervals. The analysis area was 700  $\mu\text{m}$  by 300  $\mu\text{m}$ .  
418 Two areas were analysed for each sample, collecting the data in duplicate. Charge  
419 neutralisation was used with intention of preventing excessive charging of the samples during  
420 analysis. The data collected were calibrated in intensity using a transmission function  
421 characteristic of the instrument (determined using software from NPL) to make the values  
422 instrument independent. The data were then be quantified using theoretical Schofield relative  
423 sensitivity factors. The data were calibrated for binding energy using the main carbon peak C  
424 1s at 285.0 as the reference peak, and correcting all data for each sample analysis accordingly.

#### 425 **Raman spectroscopy analysis of SeNPs**

426 Aliquots of 2  $\mu\text{L}$  of SeNPs suspended in water were transferred onto a calcium fluoride  
427 ( $\text{CaF}_2$ ) slide and air-dried prior to Raman analysis. Raman spectra were obtained using a  
428 Horiba LabRam HR and a modified Horiba LabRam HR (Wellsens Biotech. Ltd., China).  
429 Three factors have been modified in this new Raman system to improve Raman spectral  
430 quality. These comprise shortening the Raman light path, employing a low noise and  
431 sensitive EMCCD for the Raman signal detection, and increasing incident laser power. The  
432 old and new modified systems are identical except these three factors. The Raman signals  
433 were collected by a Newton EMCCD (DU970N-BV, Andor, UK) utilizing a  $1600 \times 200$   
434 array of 16  $\mu\text{m}$  pixels with thermoelectric cooling down to  $-70^\circ\text{C}$  for negligible dark current.  
435 A 532 nm Nd:YAG laser (Ventus, Laser Quantum Ltd., UK) was used as the light source for

436 Raman measurement. A 100× magnifying dry objective (NA = 0.90, Olympus, UK) was used  
437 for sample observation and Raman signal acquisition. A 600 line/mm grating was used for  
438 the measurements, resulting in a spectral resolution of  $\sim 1 \text{ cm}^{-1}$  with 1581 data points. The  
439 laser power on sample was measured by a laser power meter (Coherent Ltd.). The Raman  
440 spectra were processed by background subtraction (using spectra from cell free region on the  
441 same slide) and normalization using the Labspec5 software (HORIBA Jobin Yvon Ltd., UK).

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

443 In order to determine the functional groups present on the SeNPs, the FTIR spectra of SeNPs  
444 were recorded on a PerkinElmer Spectrum 100 FT-IR Spectrometer equipped with an  
445 attenuated total reflectance (ATR) attachment. Spectra were recorded from 4,000 to 650  $\text{cm}^{-1}$ ,  
446 and 4 scans were averaged at a resolution of 4  $\text{cm}^{-1}$ . Extracted SeNPs were freeze dried  
447 overnight and analyzed without further treatment. For comparison, the FTIR spectra of  
448 samples of bacterial cells (as control) and chemically synthesized SeNPs (Chem-SeNPs) were  
449 also recorded. For the controls, freshly grown cultures ( $\text{OD}_{600} \sim 0.7$ ) of *Mc. capsulatus* (Bath)  
450 were centrifuged at  $11000 \times g$  for 10 min to obtain the cell pellets. The pellets were washed  
451 twice with phosphate buffered saline (Sodium chloride, 150 mM, and sodium phosphate, 150  
452 mM) pH 7.2, and then freeze dried overnight. The synthesis of Chem-SeNPs was done  
453 according to the procedure of (63) as follows: 1.0 mL of 50 mM L-cysteine (Sigma-Aldrich,  
454 Dorset, UK) solution was added dropwise into 1.0 mL of 0.1 M  $\text{Na}_2\text{SeO}_3$ . The mixed solution  
455 was then stirred for 30 min at room temperature. The Chem-SeNPs were pelleted by  
456 centrifugation (at  $15000 \times g$ ; 10 min), and then freeze dried overnight.

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## 463 **Conflict of Interest**

464 The authors declare no conflict of interest.

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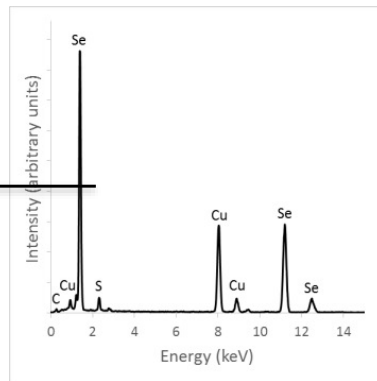
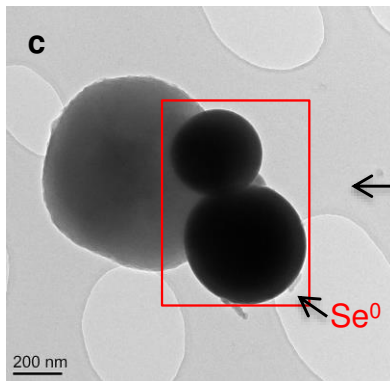
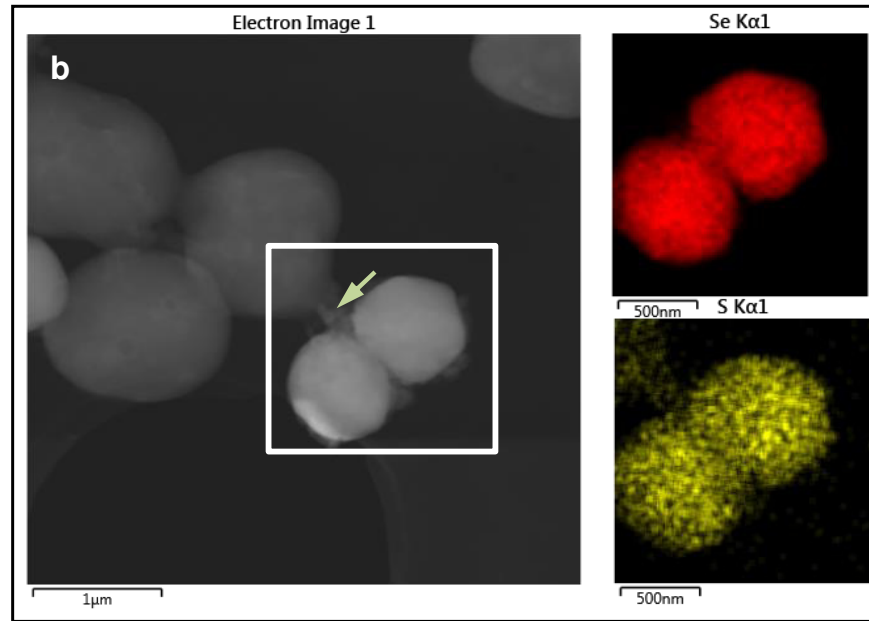
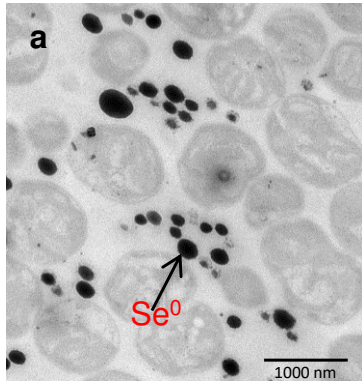
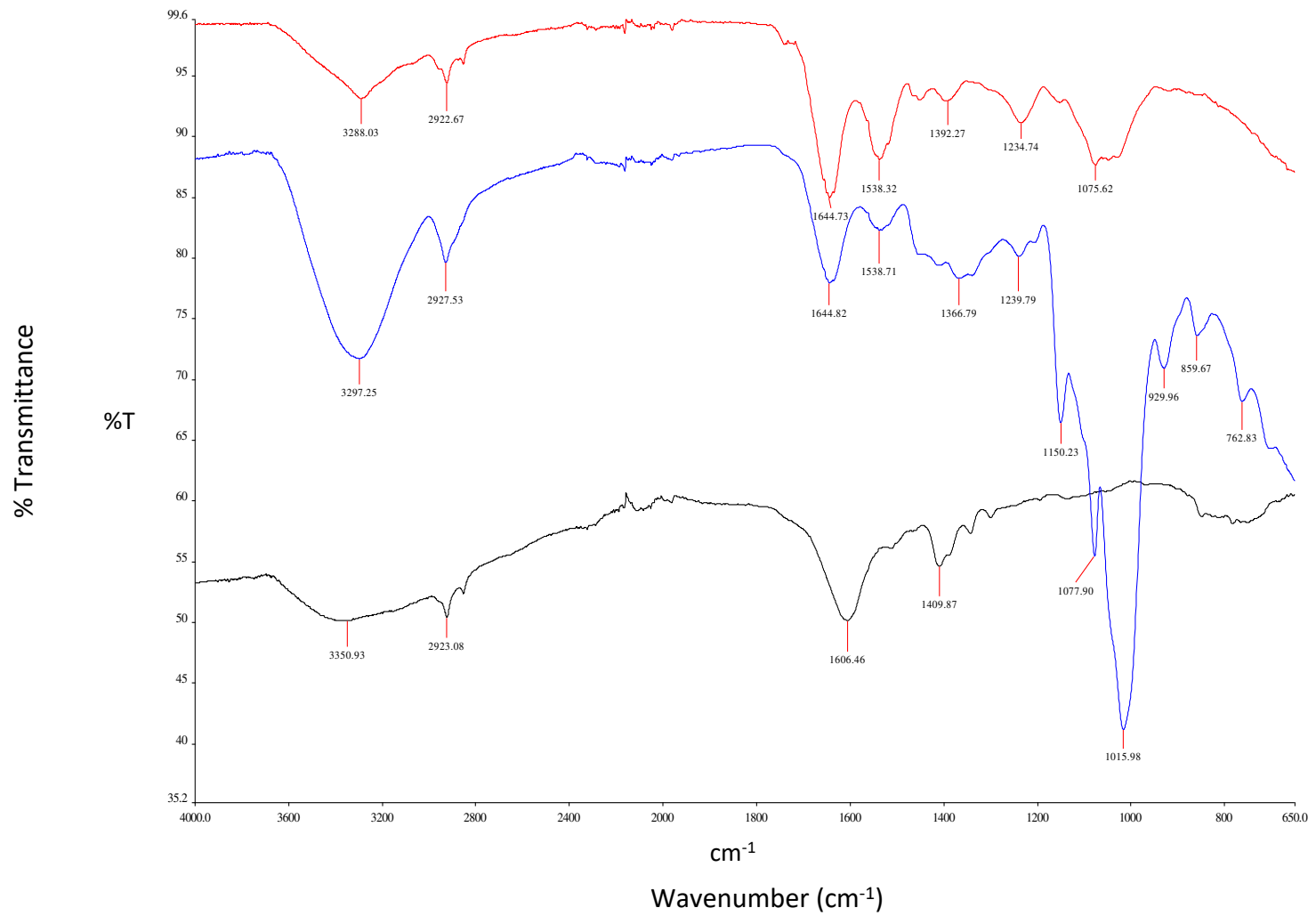


Figure2



**Figure 3**

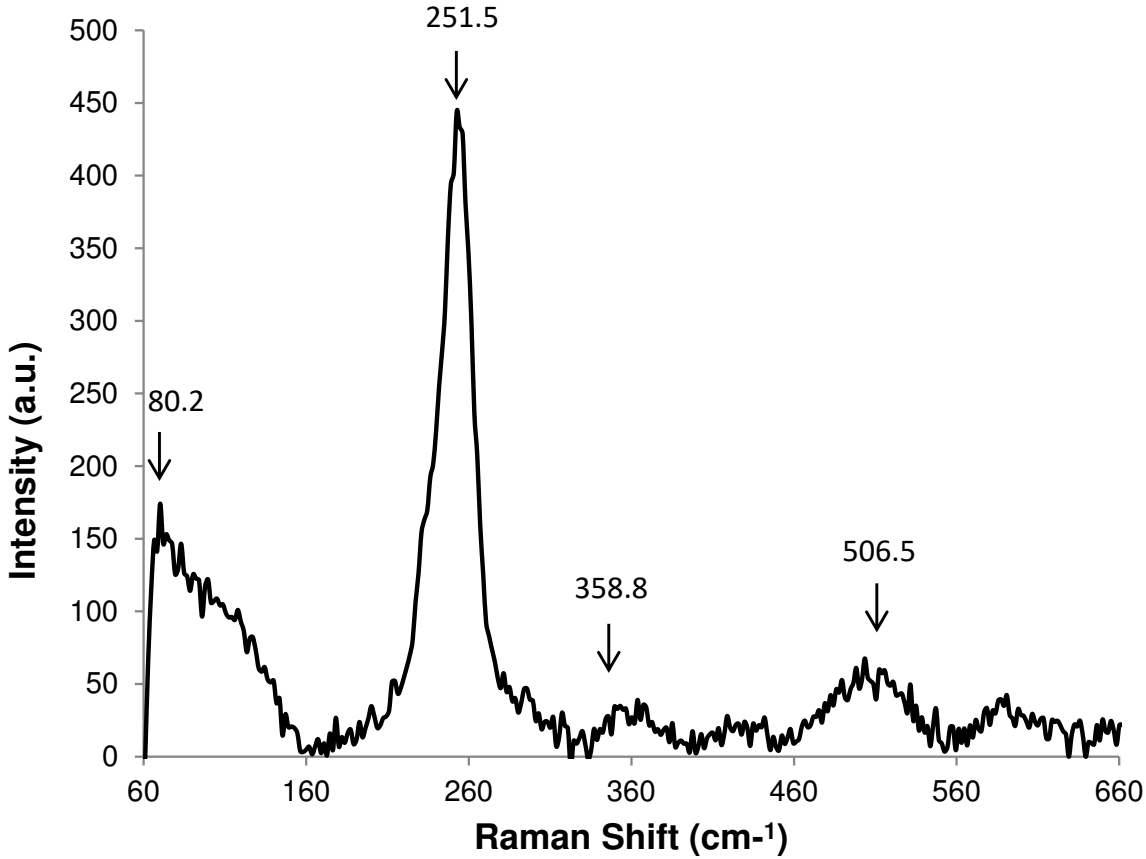


Figure 4

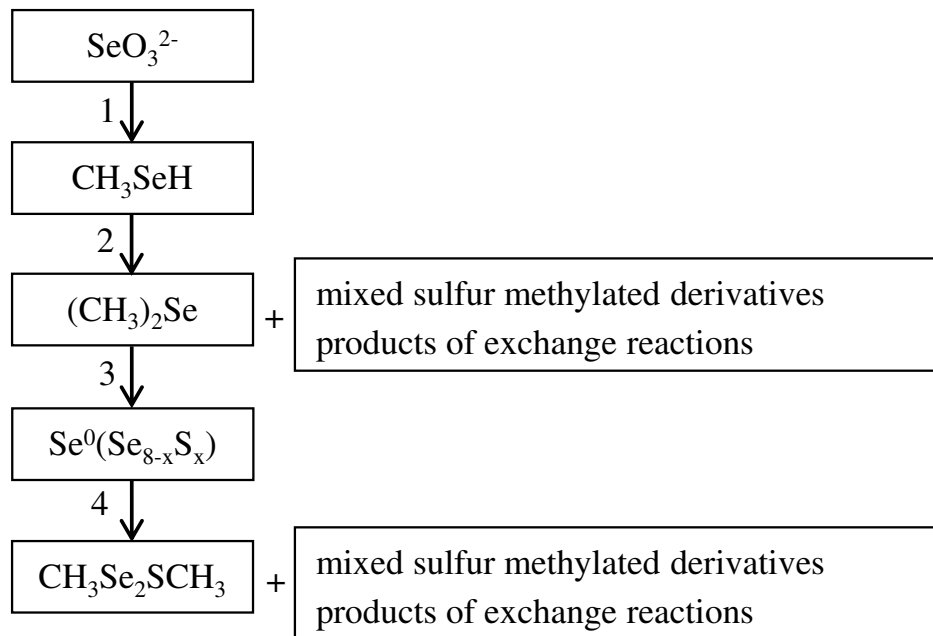
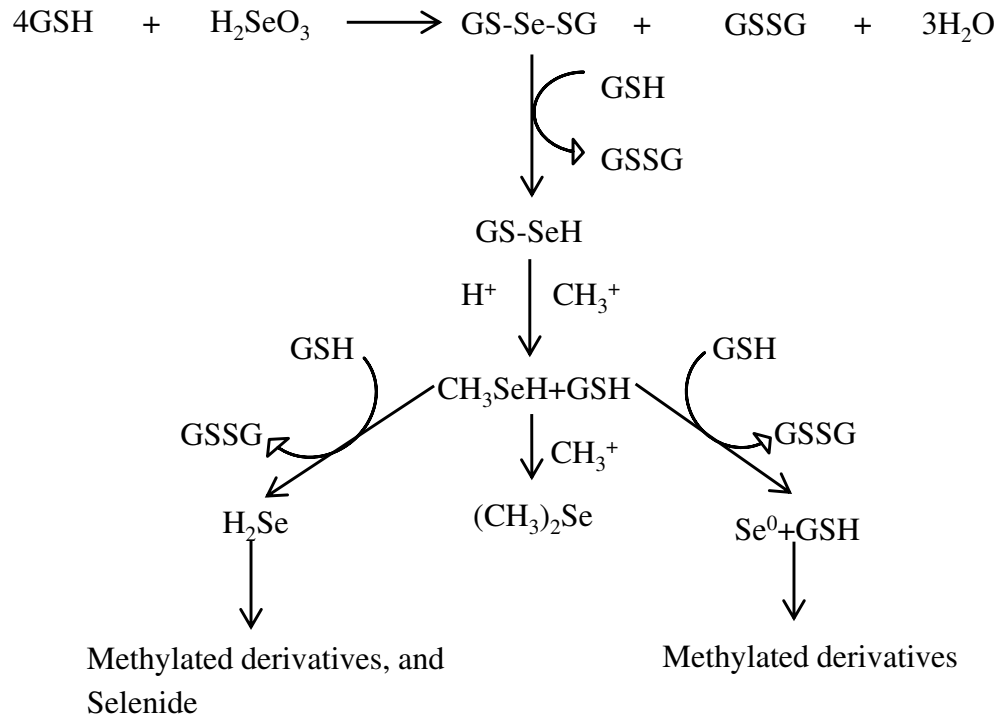


Figure 5



## List of Figures

**Figure 1** TEM thin-section micrographs of *Mc. capsulatus* (a) exposed to 20 mg L<sup>-1</sup> SeO<sub>3</sub><sup>2-</sup>, showing the extracellular locations of the Se<sup>0</sup> 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<sup>-1</sup> SeO<sub>3</sub><sup>2-</sup> (c) with EDX analysis within the electron dense regions (Se<sup>0</sup> nanospheres). The green arrow in part (b) indicates the “trail” of sulfur-containing material between the cells and the particles which may indicate partly particle-associated proteinaceous material derived from the cells. Cells were fixed with 3% glutaraldehyde and 2% OsO<sub>4</sub> 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<sup>-1</sup> SeO<sub>3</sub><sup>2-</sup> and harvested at OD<sub>600</sub> ~ 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 Na<sub>2</sub>SeO<sub>3</sub> with L-cysteine. The spectra are representatives of 5 runs of the experiments.

**Figure 3** The Raman spectra of purified Se nanospheres from *Mc. capsulatus*

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

**Figure 5** A schematic diagram showing the formation of methyl selenol, selenium particles and methylated derivatives.

## List of Tables

Table 1 Tentative assignments of main bands to the relevant functional groups (wavenumber,  $\text{cm}^{-1}$ ) (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 ( $\text{COO}^-$ )	Amide II (in proteins)	$-\text{CH}_2-\text{CH}_3$ (in proteins, lipids, polyesters, etc.)	C=O of ( $\text{COO}^-$ )	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. The control is the same medium with no added selenite.

Incubation Time		Species										
		Methyl selenol	DMS <sub>2</sub> S	DMDSe	Bis (Methylseleno) methane	Dimethyl selenosulfide	Dimethyl diselenenyl sulfide	Triselenothione/ Dimethyltriselenide	Benzothiazole	Diethyl sulfoxide	Propanesulfonyl	Dodecanethiol
In solution (selenite amended)	4 h	+	+	+	+	-	+	-	+	-	-	-
	20 h	+	+	+	+	+	+	+	+	+	-	-
In headspace (selenite amended)	4 h	+	+	+	-	-	-	-	+	-	-	-
	20 h	+	+	+	-	-	-	+	+	-	-	-
(Control)	20 h	-	-	-	-	-	-	-	+	-	+	+

The signs denote detected (+) and undetected (-).