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Selenium Analysis in Waters

Part 2: Speciation Methods

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In aquatic ecosystems, there is often no correlation between the total concentration of selenium present in the water column and the toxic effects observed in that environment. This is due, in part, to the variation in the bioavailability of different selenium species to organisms at the base of the aquatic food chain. The first part of this review (Kumkrong et al., 2018) discusses regulatory framework and standard methodologies for selenium analysis in waters. In this second article, we are reviewing the state of speciation analysis and importance of speciation data for decision makers in industry and regulators. We look in detail at fractionation methods for speciation, including the popular selective sequential hydride generation. We examine advantages and limitations of these methods, in terms of achievable detection limits and interferences from other matrix species, as well as the potential to over- or under-estimate operationally-defined fractions based on the various conversion steps involved in fractionation processes. Additionally, we discuss methods of discrete speciation (through separation methods), their importance in analyzing individual selenium species, difficulties associated with their implementation, as well as ways to overcome these difficulties. We also provide a brief overview of biological treatment methods for the remediation of selenium-contaminated waters. We discuss the importance of selenium speciation in the application of these methods and their potential to actually increase the bioavailability of selenium despite decreasing its total waterborne concentration.

Highlights - chemical speciation plays a vital role in selenium toxicity in aquatic systems

- biological treatment methods can alter selenium speciation in waters and effluents

- sub-ng/L detection limits can be obtained for selenium with several analysis methods

Keywords mass spectrometry, chromatography, solid phase extraction, selective sequential hydride generation, bioremediation

Contents

List of Acronyms **AEC** Anion Exchange Chromatography, **AAS** Atomic Absorbance Spectrometry, **AES** Atomic Emission Spectrometry, **AFS** Atomic Fluorescence Spectrometry, **CC** Collision Cell, **CD** Conductivity Detection, **CE** Capillary Electrophoresis, **CEC** Cation Exchange Chromatography, **CI** Chemical Ionization, **CID** Collision Induced Dissociation, **CPE** Cloud Point Extraction, **CSV** Cathodic Stripping Voltammetry, **CTAB** Certyl Trimethylamminium Bromide, **CVG** Chemical Vapour Generation, **DES** Deep Eutectic Solvent, **DESe** Diethyl Selenide, **DLLME** Dispersive Liquid-Liquid Microextraction, **DMDSe** Dimethyl Diselenide, **DMSe** Dimethyl Selenide, **DMSPE** Dispersive Micro Solid Phase Extraction, **DRC** Dynamic Reaction Cell, **EDXRF** Energy-Dispersive X-Ray Fluorescence Spectrometry, **EI** Electron Ionization, **ESI** Electrospray Ionization, **ETV** Electrothermal Vapourization, **FGD** Flue Gas Desulphurization, **FID** Flame Ionization Detection, **GC** Gas Chromatography, **HFLPME** Hollow Fibre Liquid Phase Microextraction, **HG** Hydride Generation, **HS** Headspace, **ICP** Inductively-Coupled Plasma, **IPRP** Ion-Pairing Reversed Phase, **LLME** Liquid-Liquid Microextraction, **LOD** Limit of Detection, **MeSeCys** Methylselenocysteine, **MS** Mass Spectrometry, **MS/MS** Tandem Mass Spectrometry, **OES** Optical Emission Spectrometry, **org-Se** Organoselenium, **PVG** Photochemical Vapour Generation, **SEC** Size-Exclusion Chromatogarphy, **SeCys** Selenocysteine, **$SeCys_2$** Selenocystine, **SEM-EDXS** Scanning Electron Microscopy-Energy Disperse X-Ray Spectroscopy, **SeMet** Selenomethionine, **SPE** Solid Phase Extraction, **SPME** Solid Phase Microextraction, **SSHG** Selective Sequential Hydride Generation, **TBAH** Tetrabutylamminium hydroxide, **TEM** Transmission Electron Microscopy, **TSe** Total Selenium, **UASB** Upflow Anaerobic Sludge Bed, **USAEME** Ultrasound-Assisted Emulsification Microextraction, **U.S. EPA** United States Environmental Protection Agency, **UV** Ultra-Violet, **VG** Vapour Generation, **XAS** X-Ray Absorption Spectrometry

1. Introduction

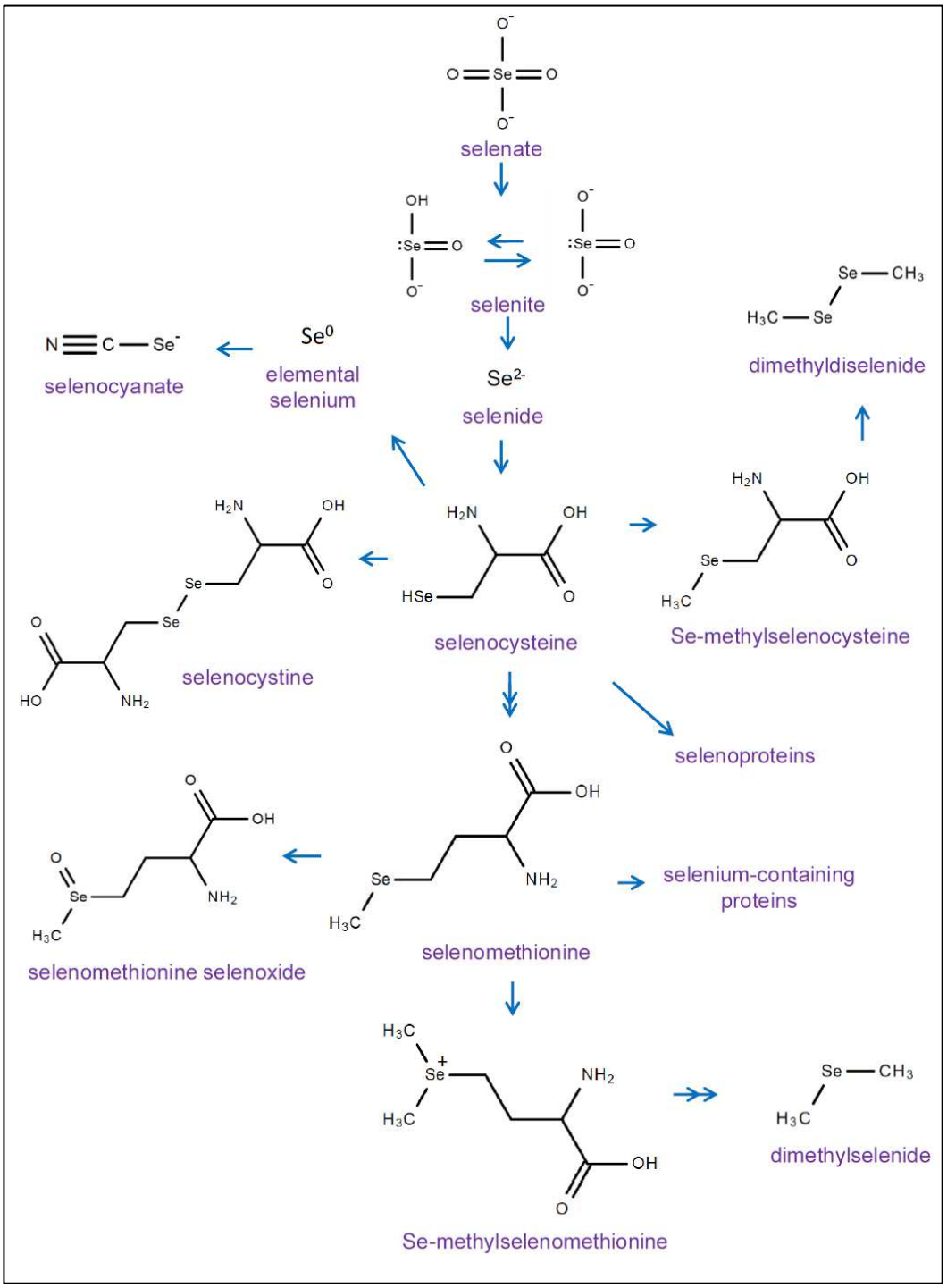
This article is the second in a two part series by these authors. The first part of this review (Kumkrong *et al.*, 2018) discusses regulatory framework and standard methodologies for selenium analysis in waters used by government and non-governmental bodies in the monitoring of these concentrations. In this second article we are reviewing the state of speciation analysis and importance of speciation data for decision makers in industry and regulators.

Selenium (Se) is a naturally occurring element, distributed unevenly in the earth's crust with a fairly low overall abundance. In animals, Se acts as an antioxidant and is required for the regulation of glutathione peroxidase as well as other biological functions. However, there is a very narrow threshold between levels of essentiality and toxicity, and environmental Se contamination has led to negative effects on aquatic ecosystems (Janz *et al.*, 2010). For this reason, Se concentrations in waters are regulated worldwide. Here, we take a closer look at the Se present in the environment, particularly in the context of treated industrial wastewaters, and discuss the need for more robust analytical methods for the analysis of Se in these waters. Specifically, we examine the role of speciation analysis as a more relevant indicator of the potential for toxic effects of Se in the environment.

2. Selenium in Aquatic Ecosystems

Selenium is released naturally into the environment from the weathering of seleniferous soils, black shales, and other sedimentary rocks of marine origin (Lussier *et al.*, 2003; Tabelin *et al.*, 2014a, b). Agricultural irrigation of alkaline seleniferous soils significantly increases the mobilization of Se, as has been seen in semi-arid regions of the southwestern United States (Ohlendorf, 2003). Se is also mobilized by mining activities relating to copper, gold, silver, coal, uranium, and phosphate (Ballard and Mines; De Gregori *et al.*, 2002; Hu *et al.*, 2009; Khamkhash *et al.*, 2017; Martell *et al.*, 2016). Additionally, Se is released into the environment as a by-product of metallurgical processes, petroleum refining, and coal

92 combustion (Maher *et al.*, 2010). Typically, high sulphur (S) areas also contain elevated levels of Se,
93 meaning fuels mined from these areas (such as the southeastern United States) are of particular
94 concern. Se is leached into waterways as the soluble oxyanions selenite (Se(IV) or $HSeO_3^-$) and/or
95 selenate (Se(VI) or SeO_4^{2-}) (Howard, 1977). The form of Se present in the water is strongly dependent on
96 water chemistry, with Se(VI) present at oxidizing conditions. In more reducing environments Se(IV)
97 dominates, generally as $HSeO_3^-$ for most natural waters, though some SeO_3^{2-} is present at about pH ≈ 8
98 and above. Elemental Se^0 can be formed at more reducing conditions, and in extremely reducing
99 environments where p*e* < -1, HSe^- may form (Khamkhash *et al.*, 2017). Once they are dissolved in the
100 water, Se oxyanions can be adsorbed to iron and aluminium oxides, clay minerals, and carbonates
101 (Francisco *et al.*, 2018; Hiemstra *et al.*, 2007; Renard *et al.*, 2013). Additionally, Se(IV) and/or Se(VI) can
102 be taken up by plants or invertebrates (Li *et al.*, 2017; Malisa, 2001; Saha *et al.*, 2017) which can cause
103 further transformations to elemental Se^0 or Se^{2-} (selenide), the latter of which is typically found as an
104 organic Se species such as selenomethionine (SeMet) or selenocysteine (SeCys) (Maher *et al.*, 2010). The
105 uptake of different Se species into the aquatic food chain varies significantly depending on the species of
106 algae present at the base of this food chain (Baines and Fisher, 2011), though in freshwater systems it is
107 accepted that green algae take up SeMet at a significantly greater rate than the inorganic oxyanions
108 (Besser *et al.*, 1989; Riedel *et al.*, 1991; Sandholm *et al.*, 1973). Figure 1 provides an overview of some of
109 Se species which could potentially be found in aquatic environments, and Table 1 shows concentrations
110 of Se in selected environments.



111

112 **Figure 1**: Selected inorganic and organic Se species. Arrows denote the metabolic pathway taken by

113 plants, based on data from Pilon-Smits and Quinn (2010); selenocyanate formation based on Simmons

114 and Wallschläger (2011). Double arrows indicate the condensation of several metabolic steps. Structures

115 drawn using the Ketcher Chemical Structure Editor (Life Sciences Open Source, 2014).

Table 1: A Non-Exhaustive List of Se Concentrations in (Untreated) Contaminated Waters

Site / Source	Se Concentration (µg/L) [a]	Cause	Reference
Groundwater, Sirmaur District, Himachal Pradesh, India	Average : 689 ± 1480 Median : 6.25 Range : 1 - 4475	industrial activity	(Kashyap *et al.*, 2018)
River Water, Lepelle, Botshabelo, and Diphuti, South Africa	Average : 207.82	mining, coal-fired power generation, miscellaneous industry, agriculture	(Genthe *et al.*, 2018)
Lower Fountain Creek, Colorado, USA (Lowest Concentration Sample Site)	TSe : 0.102 ± 0.011 Se(IV) : 0.226 ± 0.020 Se(VI) : 0.0003 ± 0.031	geological source (se-rich pierre shale)	(Carsella *et al.*, 2017)
Lower Fountain Creek, Colorado, USA (Highest Concentration Sample Site)	TSe : 10.778 ± 0.142 Se(IV) : 0.51 ± 0.115 Se(VI) : 10.075 ± 0.110	geological source (se-rich pierre shale)	(Carsella *et al.*, 2017)
Produced Water from Hydraulic Fracking, California, USA	Average : 1900 ± 3300 Median : 520 Range : 29 - 17000	mobilization of deep geological deposits by hydraulic fracking	(Chittick and Srebotnjak, 2017)
Groundwater, Nawanshahar District, Punjab, India	Average : 1.83 ± 2.77 Range : 0.07 - 12.3	geological deposits	(Dhillon and Dhillon, 2016)
Chasicó Lake, Buenos Aires, Argentina	75.9 ± 2.7	geological deposits, mobilized by agriculture and industry	(Avigliano *et al.*, 2015)
Porcupine River, Timmins, Ontario, Canada	TSe : 16.8 ± 0.4 Se(IV) : > 15.9 [(b)] Se(VI) : < 0.9 [(b)] $SeCN^-$: 0.215 ± 0.010 org-Se : < 0.2	metallurgical facility	(LeBlanc *et al.*, 2012)
Refinery Effluent (USA)	Se(IV) : 180	industrial	(Spacil *et al.*, 2011)
Groundwater wells, Kelheim, Lower Bavaria, Germany	TSe : 12.5 - 325.6 Se(IV) : < 5 - 19.7 Se(VI) : 10.5 - 328	high-se pyrite and iron oxide deposits used during construction of roads and railways	(Raessler *et al.*, 2000)
Pore-waters from Mannering Bay, Lake Macquarie, New South Wales, Australia	1.86 ± 1.5	fly ash from a coal power plant	(Peters *et al.*, 1999)
San Luis Drain, California, USA (1983)	290 - 310	agricultural drainage from seleniferous soil	(Saiki and Lowe, 1987)
Kesterson Ponds, California, USA (1983)	36 - 320	agricultural drainage from seleniferous soil	(Saiki and Lowe, 1987)

(a) Total Se unless otherwise stated

(b) Concentrations within the river's algal bloom; upstream of the bloom Se was approximately 50 % Se(IV), 50 % Se(VI)

Se is considered an essential micronutrient for animals, though in general cases of deficiency are quite rare. This essentiality is related to the specific incorporation of Se, as SeCys, into selenoproteins, which is genetically encoded by the UGA (stop) codon (Pilon-Smits and Quinn, 2010; Stadtman, 1996). These proteins perform a variety of functions, including catalysis of oxidation-reduction reactions through the action of glutathione peroxidases and thioredoxin reductases, the latter of which play a role in the defense against oxidative stress, as well as in DNA synthesis and protein repair (Janz *et al.*, 2010). Research into the function of other selenoproteins is ongoing – while 25 selenoproteins have been identified in the human selenoproteome (Kryukov *et al.*, 2003), for example, some of their functions remain undefined (Rayman, 2012).

There is a narrow range between Se essentiality and toxicity, making the latter the more common environmental scenario (Young *et al.*, 2010). While there is still some debate regarding the mechanisms of Se toxicity, it does appear to occur through an oxidative stress mechanism. Through a reaction catalyzed by glutathione peroxidase, Se has been shown to increase the ratio of oxidized to reduced glutathione, increasing oxidative cell damage (Hoffman, 2002). Se speciation plays an important role here because not all forms of Se are equal in their ability to induce oxidative stress. SeMet, for example, does not react strongly with glutathione (Spallholz and Hoffman, 2002). However, *in vivo* metabolism of SeMet to methylselenol has been observed, and since there latter is more reactive towards glutathione, toxic effects have been noted following exposure to SeMet (Janz *et al.*, 2010; Palace *et al.*, 2004). Conversely, some plant species have the ability to detoxify Se. Both Se^0 (Van Hoewyk *et al.*, 2005) and Se-methylselenocysteine (MeSeCys) (Neuhierl *et al.*, 1999) have been noted to be formed by plants during the metabolism of Se, and these species can be accumulated without toxic effects. MeSeCys is often formed as an intermediate during the production of the volatile species dimethyl diselenide (DMDSe), which is excreted; another volatile species, dimethyl selenide (DMSe) is formed via the metabolisms of SeMet (Pilon-Smits and Quinn, 2010).

Se toxicity is a concern in aquatic ecosystems due to its ability to bioaccumulate in the aquatic food chain, with the greatest degree of bioconcentration occurring between the water and lowest tropic level (Baines and Fisher, 2011). As it is a reproductive toxin, the teratogenic effects of Se are most commonly observed in oviparous vertebrates such as predatory fish and waterfowl living in aquatic environments (Hamilton and Buhl, 2003; Hamilton, 2004; Hume, 2014; Janz *et al.*, 2010; May *et al.*, 2008; Muscatello *et al.*, 2008).

With concerns about environmental Se toxicity increasing, particularly in North America, there has been much focus on the remediation of contaminated waters through various approaches including physical, chemical, and biological treatment methods. Over the past few decades, biological treatment methods have been receiving much attention due to their relatively low cost and high efficiency (Dungan and Frankenberger, 1999), but have the side-effect of altering Se speciation in their effluents in potentially unpredictable manners. Specifically, the ability of algae and bacteria to produce and release organic Se species (SeMet, particularly) (LeBlanc and Wallschläger, 2016), means that despite the overall efficiency with which the Se is removed from waters by these biological treatment systems, the Se in the effluents may actually be more bioavailable than what was present initially (Amweg *et al.*, 2003).

It is apparent that an understanding of the Se speciation in an aquatic system could play an important role in establishing the potential for Se-induced toxicity because aquatic Se speciation varies widely from one ecosystem to another – inputs depend strongly on the type of source (U.S. EPA., 2016), but both abiotic and biotic factors within an ecosystem have been shown to significantly alter Se speciation (LeBlanc *et al.*, 2012; LeBlanc and Wallschläger, 2016; Neumann *et al.*, 2003). While monitoring fish tissue concentrations will allow similar conclusions to be drawn, the observation of elevated concentrations essentially means damage is already occurring in the ecosystem. Examining waterborne species (especially in effluents) may allow for predictions of such damage, and potentially for mitigation. Therefore, it is essential that analytical methods for the speciation analysis of Se are optimized to not

only distinguish between Se species, but to do so at environmentally-relevant concentrations and in the presence of interfering constituents including anions and dissolved organic matter to arrive at accurate and precise species abundance values.

Recent reviews by Tan *et al.* (2016) and Santos and coworkers (2015) provide thorough overviews of recent advances in the treatment of Se-contaminated water, and also provide several examples of Se concentrations in various types of contaminated waters. However, only Santos and coworkers (2015) briefly touch on the analytical methods involved in the speciation analysis of the Se present in such systems. Conversely, Pettine *et al.* (2015) review various analytical methods in great detail, but focus mostly on total Se analysis, with some discussion of fractionation methods (those that are based on the detection of a single species, often Se(IV)). Pyrzyńska's (1996) review discusses the speciation analysis of organic Se compounds in much more detail, but is now 22 years old and is therefore missing the most recent advances in this field. In the present review, we critically examine various analytical methods capable of evaluating Se speciation in natural waters and wastewaters, particularly focussing on advances from the past two decades. We then discuss some bioremediation techniques and the importance of Se speciation both before and after treatment.

3. Fractionation Methods for Selenium Speciation

Among the most basic speciation methods are those that take advantage of differences in the physical and chemical behaviours of various Se species. Among these behaviours are the ability to form volatile species for direct analysis through spectrophotometric methods, the potential for complexation of a single species with another chemical agent (resin or dissolved chelator), and the formation of a solid precipitate either through the application of an electric potential or the addition of another chemical species to solution. The inherent difficulty with these types of method lies in the fact that they result in "operationally-defined" fractions and often combine several distinct Se species into a single category –

most notably, this is the case for organic Se, which is distinguished from Se(IV) and Se(VI), but is not defined in further detail (with a few exceptions in which a "selenoamino acid-like" fraction is differentiated from the sum of organic Se, as explained in Section 3.5). Additionally, such types of analyses are vulnerable to biases as they are often based on the assumption that only specific Se species are reactive to the applied method, which can result in systematic over- or under-estimations of the actual proportions of Se in particular fractions. Unlike separation methods for speciation analysis (discussed in Section 4), fractionation methods typically measure a "total Se" (TSe) amount, relying on differences between this and other measured fractions to account for additional species rather than analyzing them directly.

Below, several analysis methods using chemical fractionation for Se speciation are discussed in detail, with particular emphasis on detection limits. The reader should keep in mind that these methods' usage are *operationally-defined* fractions for the characterization of Se in environmental samples, especially with regard to their potential advantages or limitations.

3.1 Selective Sequential Hydride Generation

Perhaps the most well-known and most widely-applied method of Se speciation through fractionation is selective sequential hydride generation (SSHG), which takes advantage of the fact that Se(IV) forms volatile SeH_2 in the presence of borohydride (BH_4^-). Various redox chemistries are then employed as sample preparation methods prior to HG to differentiate between various Se species in solution.

The original Se speciation studies employing HG took advantage of the fact that only Se(IV) forms H_2Se under acidic conditions in the presence of BH_4^- as described by Cutter (1976) and Mester, D'Ulivo, and coworkers (D'Ulivo *et al.*, 2005; Meija *et al.*, 2006). At the optimal HCl concentration of 4 mol/L determined by McDaniel *et al.* (1976), Cutter (1976) observed no detectable signal in a 0.2 µg/L Se(VI) standard, though an equivalent concentration of Se(IV) was easily detected by atomic absorption

spectrometry (AAS) following HG. However, a prolonged heating of the acidified sample prior to mixing with BH_4^- was shown to quantitatively (98%) reduce Se(VI) to Se(IV); the required heating time was shown to vary by sample size, with freshwaters requiring slightly longer heating than seawaters, though exceeding this optimal maximum caused further reduction of Se(IV) to Se^0 (which is not detected by HG). Furthermore, the addition of a UV irradiation step to decompose organic Se species (-II) in a photo-oxidation process to arrive at Se(VI) prior to heating the sample (Chen *et al.*, 2005b), or the use of a microwave digestion process for similar purposes (Velinsky and Cutter, 1990), was employed to produce a "total Se" measurement. This sequential procedure allowed researchers to determine the three operationally-defined fractions typically discussed: Se(IV) (direct HG analysis), Se(VI) (['Se(IV) + Se(VI)' fraction] – Se(IV)), and Se(-II) ('total Se' – ['Se(IV) + Se(VI)' fraction]).

The SSHG method was further optimized by Chen, Belzile, and coworkers (2005a; 2005b) following a thorough study of the photochemical behaviours of several inorganic and organic Se species in waters containing various other ions. Based on the observed decomposition of several org-Se species (SeMet, MeSeCys, $SeCys_2$, and selenourea) following heating in 3.0 mol/L HCl, the method discussed above was altered to account for the potential for overestimation of the Se(VI) content. Due to the fact that oxidation of Se(IV) was blocked during UV irradiation in a 1 % HNO_3 matrix in the presence of 2 % HCl, this treatment was applied as a second step in the SSHG process, arriving at a 'Se(IV) + org-Se' fraction. In a final step to determine total Se, the irradiated sample was acidified and heated to reduce Se(VI) to Se(IV) for HG analysis.

Table 2: Examples of Fractionation Methods for Se Speciation

Method	Fractions Analyzed[c]	Detection Limit(s)[d] (ng Se/L)	Working Range(s)[f] (µg Se/L)	Sample Type(s), Spike Recoveries (%)	Ref.
HG-AFS	Se(IV) Se(IV) + Se(VI) Total Se	Se(IV) : 50 Se(VI) : 60 Org-Se : 60	0.5 - 15.0	[b]**Tap Water**: Se(IV): 93.5 - 102.6, Se(VI) : 94.7 - 97.3 [a]**FGD Water**: Se(IV) : 95.5 - 101.5 Se(IV) + Se(VI) : 93.5 - 96 Total Se : 93.5 - 101.5	(Zhong *et al.*, 2011)
HG-AFS	Se(IV) Se(IV) + org-Se Total Se	5	0.005 - 10,000	[a]**Lake Water**: Se(IV) : 80 - 110 Se(VI) : 80.4 - 107.1	(Chen *et al.*, 2005a; Chen *et al.*, 2005b)
PVG-AFS	Se(IV) Se(IV) + Se(VI)	Se(IV) : 100 [e] Se(VI) : 100 [e]	0.1 - 100	[b]**Wastewater:** Se(IV) : 90 -110 Se(VI) : 80 - 115	(Zheng *et al.*, 2008)
PVG-ICP-MS	^{77,78,82}Se(IV) [^{77,78,82}Se(IV) + ^{77,78,82}Se(VI)]	Se(IV) : 20 Se(VI) : 20	0.02 - 100		
MSPE-ICP-MS	^{82}Se(IV) ^{82}Se(IV) + ^{82}Se(VI)	0.094	n/a	[a]**Agricultural Water** Se(IV) : 94.5 - 103.3 Se(VI) : 98.0 - 99.1	(Huang *et al.*, 2012a)
DMSPE-EDXRF	Se(IV) Se(IV) + Se(VI)	32	0.032 - 500	[a,b]**Mineral Water:** Se(IV) : 98 Se(VI) : 99 Se(IV) + Se(VI) : 94 + 97	(Kocot *et al.*, 2015)
CP-FI-HG-ICP-OES	Se(IV) Se(IV) + Se(VI)	30	0.03 - 200	[a]**River Water:** Se(IV) : 98.00 - 104.00 Se(VI) : 95.33 - 104.00	(Escudero *et al.*, 2015)
UALPME-DES-ETV-AAS	Se(IV) Se(IV) + Se(VI)	4.61	0.2 - 8	[b]**Mineral Water / Tap Water:** Se(IV) : 97 - 99 Se(VI) : 96 - 99	(Panhwar *et al.*, 2017)
DLLME-ETV-ICP-MS	^{77}Se(IV) ^{77}Se(IV) + ^{77}Se(VI)	8.6	0.003 - 10	[a]**Lake, River Water:** Se(IV) : 86.3 - 96.7 Se(VI) : 86.7 - 94.1	(Liu *et al.*, 2015)
DLLME-SFOD-UV/Vis	Se(IV) Se(IV) + Se(VI)	1600	5-600	[b]**River, Sea Water:** Se(IV): 96.0 - 101.0 Se(VI) : 95.0 - 97.8	(Dadfarnia *et al.*, 2014)
CPE-ETV-ICP-MS	^{82}Se(IV) ^{82}Se(IV) + ^{82}Se(VI)	50	0.25 - 50	[a]**Lake, River Water:** Se(IV) : 102 - 109 Se(VI) : 104	(Li *et al.*, 2008)
CPE-ETV-AAS	Se(IV) Se(IV) + Se(VI)	2.5	-	[b]**Tap, Groundwater:** Se(IV) : 98 - 110 Se(VI) : 92 - 108	(Sounderajan *et al.*, 2010)
HF-LPME-ETV-AAS	Se(IV) Se(IV) + Se(VI)	Se(IV) : 5 Se(VI) : 6	0.005 - 35	[b] **River, Sea Water /** [b]**Wastewater** Se(IV) : 94 – 103 / 99 - 102 Se(VI) : 94 – 99 / 102 - 105	(Ghasemi *et al.*, 2010)

[a]species spiked individually, [b]species spiked simultaneously, [c] isotopes noted for MS methods, [d] when only one value is given, LOD is based on the species detected (i.e. H_2Se from Se(IV) in HG-AAS), [e] LOD using acetic acid (same PVG method as ICP-MS detection), [f] the calibration range is listed where the authors did not state the upper end of the linear range

238 Generally, analysts use some variation of one of the methods described above – typically, a method
239 more closely resembling Cutter's (1976), sometimes utilizing different methods for the reduction of
240 Se(VI) to Se(IV) or for the decomposition of organic Se species. Zhong *et al.* (2011), for example,
241 determined total Se following a 120°C digestion in 40% HCl, followed by reduction to Se(IV). This
242 method achieved detection limits of 50 to 60 ng/L and exhibited good spike recoveries in various water
243 samples (Table 2). Other methods include the use of potassium persulphate for oxidation of organic Se
244 to Se(IV), or for complete oxidation of organic Se and Se(IV) to Se(VI), which is then followed by a
245 reduction step (Zhang and Frankenberger, 2003).

246 Clearly, methods described above are quite labour-intensive, requiring significant sample preparation
247 prior to the analysis of each operationally-defined fraction. Therefore, the development of online
248 analysis methods was the ideal addition to this procedure. Through the use of flow injection, multiple
249 pumps and several mixing valves and loops, this online method involves the addition of BH_4^-, sometimes
250 after the pre-reduction of Se(VI) to Se(IV), through the use of a heated and acidified mixing loop
251 (Wallschläger and Bloom, 2001) or online microwave digestion process (He *et al.*, 1998; Moreno *et al.*,
252 2000). Generally, this type of online method is used for the speciation analysis of inorganic Se only, but
253 the addition of a UV lamp could allow for the analysis of org-Se species as well, as is discussed below in
254 the context of chromatographic analysis (Darrouzès *et al.*, 2008).

255 A significant potential for error during SSHG analysis is due to the possible presence of volatile selenides
256 such as DMSe and DMDSe in natural water samples (Moreno *et al.*, 2003). SSHG analyses often do not
257 consider these species, leaving room for an overestimation of the Se(IV) fraction. To account for this,
258 Cutter (1976) modified his SSHG method by placing a gas chromatograph (GC) between the gas-liquid
259 separator and AAS, allowing for the separation of SeH_2 from DMSe and DMDSe prior to detection. Such
260 a setup allowed these volatile selenides to be quantified simultaneously – an environmentally- and

analytically-relevant endeavour. It has been suggested that DMSe and DMDSe do not reach the detector in their native form, but actually react with borohydride in the acidic medium to form more volatile species such as methane selenol (Amouroux and Donard, 1997; D'Ulivo *et al.*, 1994). Moreno *et al.* (2003) have confirmed this production of a more volatile compound (though not its identity) based on the observation that with all else being equal, the absence of sodium borohydride caused the complete disappearance of a Se signal for samples containing DMSe and DMDSe.

One fairly major complication associated with the analysis of waters (particularly contaminated wastewaters, as are typically of interest in the discussion of environmental Se) is the interference caused by the presence of transition metals, particularly Ni, Co, and Cu. These metals may interact with BH_4 causing reduced conversion to the detectable hydrides, and can act as a catalyst in the decomposition of any hydrides that are formed (Dedina and Tsalev, 1995). There are a few methods by which these issues can be resolved to allow for the analysis of Se in these types of waters. The interferences can be removed using a sample pre-treatment step by precipitation or adsorption during a column cleanup, or (more practically) the Se can be removed and concentrated by solid or liquid phase extraction as is discussed in more detail in Sections 3.3 and 3.4. Conversely, Se vapour can be produced via different approaches which do not involve borohydride (Guo *et al.*, 2003b).

3.2 Photochemical Vapour Generation

Ultraviolet (chemical) vapour generation (UV-VG or UV-CVG), also referred to as photochemical vapour generation (PVG), works on principles similar to the chemical hydride generation processes described above, wherein a volatile Se species is produced from Se(IV) in solution, separated from the liquid, and subsequently detected by some element-specific detector (such as AAS or atomic fluorescence spectrometry (AFS)), or by gas chromatography coupled to mass spectrometry (GC-MS) in some scenarios. Here, the combination of a UV lamp and the presence of certain organic species, particularly

(low molecular-weight) organic acids, causes photoreduction and formation of chemical vapour from Se(IV) (Guo *et al.*, 2003b; Zheng *et al.*, 2008). The volatile products of PVG of inorganic Se were identified when they were cryogenically trapped for subsequent analysis by GC-MS, and it was noted that the species formed varied depending on the organic acid present. Using formic acid, the two species observed were SeH_2 (60 - 70 % yield) and SeCO (30 - 40 %), whereas DMSe was formed in the presence of each of acetic and malonic acid, while diethyl selenide (DESe) was formed in solutions containing propionic acid (Guo *et al.*, 2003a).

Like with HG, the reactions described above are generally assumed only to occur for Se(IV); again, this can be exploited for speciation analysis. Pre-reduction can be used to convert Se(VI) and/or organic Se to Se(IV) prior to analysis, or this process can be conducted online. For example, Wang *et al.* (2004) developed an online reduction where, under UV-irradiation and in the presence of nano-TiO_2, Se(VI) was reduced and formed H_2Se. Further investigations by Zheng *et al.* (2008) demonstrated that this process could be quenched by cooling the system, allowing for the detection of only Se(IV) in a mixed sample. As seen in Table 2, the detection limit of this PVG-inductively-coupled plasma mass spectrometry (ICP-MS) system was quite low (20 ng/L) for a fractionation method not involving any preconcentration, and spike recoveries fell within a reasonable range.

When examining PVG methods for Se analysis, it is important to consider the potential interference from organic Se species. Chen *et al.* (2005b) have demonstrated that four organic Se species – Se-urea, SeMet, MeSeCys, and $SeCys_2$ – undergo photochemical oxidation to Se(IV) in the presence of UV light in an ultrapure water matrix, a phenomenon that is significantly enhanced in the presence of low concentrations (0.154 mol/L) of HNO_3. Similarly, UV-assisted production of volatile organic Se species has been observed upon the addition of selenoamino acids to synthetic seawater (Amouroux *et al.*, 2000). Of course, the influence of the presence of organic acids needs to be further examined before

any finite conclusions can be drawn, but these findings suggest the potential for the overestimation of inorganic Se species when organic Se is also present in a sample. Arguably, the more environmentally-relevant outcome of such an oversight would be that it would also result in an underestimation of organic Se in analyses accounting for this operationally-defined fraction as the difference between "total Se" and "Se(IV) + Se(VI)". Looking specifically to PVG, there is some evidence that these processes are occurring: Guo and coworkers (2003a) observed some production of volatile Se from the UV-irradiation of SeMet and $SeCys_2$ under these conditions, but did not investigate the identity of these species because the yield was quite low.

3.3 Voltammetry

While it is not among the most common analysis method for Se, voltammetry has been successfully employed for the speciation of Se in water samples due to the fact that, much like HG and PVG, only Se(IV) is detected with this method. Cathodic stripping voltammetry (CSV) is often employed using either a thin mercury film electrode or a hanging mercury drop electrode, resulting in the collection of Se as SeHg (Rubinskaya *et al.*, 2003) or as a copper-Se or rhodium-Se complex if Cu or Rh is added to the sample (Pettine *et al.*, 2015). Using CSV with a mercury-film electrode, Rubinskaya *et al.* (2003) achieved a detection limit of 0.1 µg/L for Se. Conversely, Bertolino and coworkers (Bertolino *et al.*, 2006) were able to detect Se at 0.004 µg/L based on a preconcentration on activated carbon, which followed the reduction of Se(IV) to Se^0 by L-ascorbic acid. Reduction of both Se(VI) and Se(VI) to Se^0 by hydrazine allowed for (inorganic) speciation analysis, where 99-104% recovery was obtained for each species in river water. There, detection was based on Osteryoung square-wave voltammetry using a platinum electrode.

3.4 Liquid-Liquid (Micro)Extraction with Complexation

Another method for the fractionation of Se species in a mixed solution involves the selective extraction of one of those species into a second liquid phase, typically after the formation of a complex between an added chemical agent and the Se species of interest, known as liquid-liquid microextraction (LLME).

A common process is the formation of piazselenols through the complexation of phenylenediamines with Se(IV) under acidic conditions. While these yellow-coloured complexes can be examined by UV/Vis spectrophotometry, detection limits are quite high when the aqueous samples are analyzed directly and it is therefore beneficial to extract the piazselenols into an organic phase. Dadfarnia *et al.* (2014) employ a variation of this method where they form a complex between Se(IV) and 3,3'-diaminobenzidine and extract the resulting piazselenols into 1-undecanol prior to measuring the absorbance at 434 nm. While detection limits achieved here were still relatively high (1.6 µg/L, see Table 2), this LLME method has the significant advantage of being robust enough to tolerate high salinity samples – up to 1.0 mol/L were examined here without issue (moving beyond this increases the solubility of 1-undecanol in the aqueous phase). This is particularly advantageous for many of the highly-contaminated environmental samples that are of interest for Se speciation, such as agricultural drainage waters and flue-gas desulphurization (FGD) waters.

There are various other chelating agents that can be used in a similar manner, though many of these do not formed a coloured complex and therefore require more sophisticated detection systems. For example, Liu *et al.* (2015) used diethyldithiocarbamate to form a complex with Se(IV) which was then extracted into a bromobenzene layer that was subsequently separated through centrifugation and analyzed by electrothermal vaporization (ETV) ICP-MS. Following this method, the detection limit of 8.6 ng/L was significantly lower than that observed with the spectrophotometric method described above,

352 despite the slightly lower enrichment factor of 107 (Liu *et al.*, 2015) (compared to 133 (Dadfarnia *et al.*,
353 2014)).

354 There are other methods using liquid-liquid extraction principles that have been modified for various
355 applications or to improve extraction efficiency: for example, hollow fiber liquid phase microextraction
356 (HFLPME), which was first described by Pedersen-Bjergaard and Rasmussen (1999). Here, a porous
357 hollow fibre is coated with an organic solvent that is immiscible in the aqueous phases, filled with an
358 "acceptor solution" and placed into the sample, which is stirred. The analyte moves through the organic
359 membrane into the acceptor solution in the hollow fibre, becoming more concentrated and leaving
360 other (non-miscible) contaminants behind in the starting sample ("donor solution").

361 The use of HFLPME is particularly advantageous for samples with low Se concentrations as it has the
362 ability to achieve high enrichment factors due to the low volume of acceptor solution used in the hollow
363 fiber. The low detection limits achieved by Ghasemi and coworkers (2010) noted in Table 2 were
364 obtained due to the enrichment factor of 480 observed in their experiment, where they first chelated
365 Se(IV) with ammonium pyrrolidinecarbodithionate before extracting the complex into toluene contained
366 in a hollow fiber. Enrichment factors ranging from 49 (Moreno *et al.*, 2013) to 410 (Xia *et al.*, 2006) have
367 also been achieved following similar HFLPME protocols.

368 Another LLME process is cloud point extraction (CPE), which is based on the principle that a
369 homogeneous solution containing a non-ionic surfactant will separate into two phases when heated to a
370 specific temperature known as the cloud point temperature. If a chelating agent is also added to
371 solution prior to heating, metal (i.e. Se) complexes can be trapped in the micelles formed during this
372 process. The layers are then separated (usually by centrifugation) and the surfactant-rich layer is
373 subsequently analysed. Since the micellular phase is present in a small volume relative to the starting

374 sample, CPE is generally associated with a good degree of preconcentration for the analyte of interest
375 (Altunay and Gürkan, 2016; Samaddar and Sen, 2014).

376 Reactions forming piazselenols can be used with CPE procedures, as has been discussed by Sounderajan
377 *et al.* (2010) who reacted Se(IV) with 3,3'-diaminobenzidine. Using the surfactant Triton X-114 (added in
378 an aqueous solution), they were able to obtain an enrichment factor of 100 following phase separation
379 and achieved detection limits of 2.5 ng/L with ETV-AAS. Similarly, the reaction of Se(IV) with
380 diethyldithiocarbamate has also been used with CPE, with the complex extracted into Triton X-114 at
381 the cloud point temperature. Here, researchers obtained a detection limit of 50 ng/L based on an
382 enrichment factor of 50 (Li *et al.*, 2008); see Table 2.

383 An interesting recent development in the field of LLME is the use of environmentally friendly extraction
384 solvents, specifically deep eutectic solvents (DES). These solvents are formed through the combination
385 of quaternary ammonium salts with hydrogen bond donors (carboxylic acids, acid amides, polyhydric
386 alcohols) in a specific molar ratio and have significantly lower freezing points than either of their
387 components (Paiva *et al.*, 2014; Panhwar *et al.*, 2017). While these solvents are more commonly used
388 for the extraction of elements and organic compounds from food (Panhwar *et al.*, 2017) or soil (Matong
389 *et al.*, 2017) samples, they have also been successfully applied in the extraction of Se(IV) complexes
390 from water samples. For example, Panhwar *et al.* (2017) used diaminobenzidine to complex Se(IV) in
391 water sample, then added a DES, sonicated the solution in the presence of a small amount (~ 2 % v/v) of
392 tetrahydrofuran to disperse the DES as nano-sized droplets in solution (increasing extraction efficiency),
393 then centrifuged the sample to separate the solvent layer. This method achieved an enrichment factor
394 of 50, and relatively low detection limits (Table 2).

395 An important common component of all of these LLME protocols is the fact that they are all selective to
396 a single Se species – typically this is Se(IV). To determine the other Se species in solution, researchers

reduce Se(VI) to Se(IV) using one of a few different methods which usually involve heating the sample to about 100°C in the presence of L-cysteine (0.5 to 1.5% w/v) (Altunay and Gürkan, 2016; Liu *et al.*, 2015) or a strong acid (HCl or HBr, usually at concentrations of 2 to 4 mol/L) (Dadfarnia *et al.*, 2014; Ghasemi *et al.*, 2010), sometimes using a microwave (Panhwar *et al.*, 2017; Sounderajan *et al.*, 2010). Following this reduction, this second sample aliquot is then subjected to the LLME procedure and the Se(VI) concentration is calculated as the difference between the two measurements. This follows the same principles discussed for SSHG, though here the "selective sequential" steps (sometimes) involve slightly different chemical reactions and an org-Se fraction is often not explicitly discussed for LLME methods.

3.5 Solid Phase (Micro)Extraction

Solid phase extraction (SPE) (sometimes referred to as solid phase microextraction (SPME)) is a common preparatory step, often used to remove chemical interferences and/or preconcentrate an analyte prior to analysis. Generally, SPE works due to interactions between the solid phase and analyte(s) (or interferences) in solution. Assuming the goal is to retain the analyte on the solid phase (the other option is to retain interfering components in the matrix solution, where the analyte is passed through the solid phase ending up in a "cleaner" solution), physical and chemical interactions cause the analyte to be ad- or ab-sorbed onto the solid phase while non-interacting matrix components are removed. After a sample is passed through the solid phase, the analyte is then eluted with a solution that causes the environment to be altered such that the analyte is no longer retained (i.e. through a pH change, or the eluent interacting more strongly with the resin and "pushing" the analyte off). Preconcentration occurs when the volume of sample loaded onto the resin is greater than the volume of eluent, if the recovery is high.

Based on the wide variety of solid phases available for purchase or which can be prepared in-house, speciation analysis can also be conducted using SPE to separate different chemical species. Most

commonly, a single species is selectively retained and concentrated, sometimes following the production of a Se-complex with an added chelating agent. The species of interest is then retained directly on the solid phase, examples of which include cetyl trimethylammonium bromide- (CTAB) modified alkyl-silica (Xiong *et al.*, 2008) and ZrO_2-modified coal cinder (Wei *et al.*, 2014). Typically, analysist will optimize the sample matrix and eluent to ensure quantitative recovery of the species of interest without interference from other Se species also present in the sample (i.e. to distinguish between Se(IV) and Se(VI)).

Recently, there has been increased interest in the application of nanoparticles to SPE procedures since their large surface area allows for significant preconcentration using only small amounts of the solid phase. For example, Nyaba *et al.* (2016) obtained an enrichment factor of 850 and a detection limit of 1.4 ng/L by combining ICP-optical emission spectrometry (ICP-OES) with their suspended dispersive SPME method using alumina nanoparticles functionalized with Aliquat-336 (which adsorbed Se(IV)). The wide variety of nanoparticles readily available also increases their versatility. Magnetic nanoparticles such as mercapto-silica-Fe_3O_4 (magnetite) can be quickly and easily separated from solution using a magnet following the adsorption of Se(IV) (Huang *et al.*, 2012a).

An interesting modification of an SPE protocol allows the retained Se to be quantified in-situ rather than after elution. In one such experiment, a Se(IV)-chelating agent complex was formed and adsorbed onto a gel-like resin, which was then mechanically separated from solution and the absorbance of the complex in the gel was measured directly by UV/Vis spectrophotometry (Amin, 2014). Due to the limited sensitivity of this type of instrumentation, detection limits for such methods can be relatively high, particularly where sample size is limited. For example, using a 25 mL sample, Amin (2014) achieved a detection limit of 2.80 µg/L following UV/Vis spectrophotometry, but using a 1000 mL sample decreased this to 0.06 µg/L, which increases the applicability of this method to analysis of natural waters where Se

443 concentrations are relatively low, but larger volumes are readily available. Conversely, Kocot *et al.*
444 (2015) used energy-dispersive X-ray fluorescence spectrometry (EDXRF) as a direct solid phase detection
445 method following dispersive micro-SPE (DMSPE) and achieved a method detection limit of 0.032 µg/L
446 due to the obtained enrichment factor of 1013 (see Table 2).

447 The combination of more than one solid phase can result in a more thorough fractionation of the Se
448 within a water sample. For example, Besser and coworkers (1994) were able to determine four Se
449 fractions – Se(IV), Se(VI), free selenoamino acids, and non-amino acid organoselenium compounds –
450 through the use of two activated charcoal micro-columns, an anion exchange micro-column, and a
451 copper-form cation exchange resin-packed column.

452 Similar to the other fractionation methods discussed thus far, SPME usually operates based on the
453 selective retention of a single Se species on the chosen resin, with the others being calculated based on
454 the difference following a reduction (or oxidation) step. However, the nature of SPE allows for another
455 option: selective elution, rather than selective retention. Since the two major Se species in natural
456 waters are anions under ambient conditions, an anion exchange resin will retain both of these species.
457 Se(VI) is retained more strongly, and will remain on the resin while Se(IV) is eluted with a lower strength
458 eluent (i.e. 0.1 mol/L HCl or HNO_3), and can be eluted in a second step with a stronger solution (Lin,
459 2007; Stripeikis *et al.*, 2004). This procedure does not account for organic Se which may pass through
460 the resin upon loading the sample, or may be weakly retained and eluted with Se(IV) causing an
461 overestimation of that fraction.

462 This selective elution process is where the (somewhat ambiguous) line between “fractionation” and
463 “speciation” methods of multi-species Se analysis can be drawn. The former often pools multiple species
464 together, whereas the goal of speciation methods is typically to examine discrete species individually.

465

4. Separation Methods for Selenium Speciation

Table 3: Examples of Separation Methods for Se Speciation

Method	Species Analyzed[(c)]	Detection Limit(s) (ng Se/L)	Working Range(s) (ng Se /L)	Sample Type(s), Spike Recoveries (%)	Ref.
USAEME-GC-FID	Se(IV) Se(VI)	Se(IV) : 50 Se(VI) : 50	200 - 35,000	[(a)]**Sea, River, Waste, Tap, Drinking Waters:** Se(IV) : 91.6 - 99.3 Se(VI) : 89.4 - 98.7	(Najafi *et al.*, 2012)
HS-SPME-GC-MS	DMSe DMDSe	DMSe : 16 DMDSe : 11	DMSe : 100 - 600,000 DMDSe : 80 - 510,000	[(b)]**Well, River, Tap, Waste Waters:** DMSe : 89.5 - 103.2 DMDSe : 93.2 - 102.7	(Ghasemi and Farahani, 2012)
SPE-AEC-CD	Se(IV) Se(VI)	Se(IV) : 800 Se(VI) : 400	4,000 - 10^6	[(b)]**Tap Water:** Se(IV) : 89.6 Se(VI) : 86.4	(Xu *et al.*, 2012)
AEC-HG-AFS	Se(IV) Se(VI) $SeCN^-$	Se(IV) : 26 Se(VI) : 33 $SeCN^-$: 34	LOD - 5,000	[(b)]**Industrial Wastwater:** Se(IV) : 85.5 - 113.5 Se(VI) : 88.0 - 108.5 $SeCN^-$: 85.3 - 113.3	(Wallschläger and Bloom, 2001)
AEC-HG-ICP-DRC-MS	^{80}Se(IV) ^{80}Se(VI) $^{80}SeCN^-$	Se(IV) : 0.15 Se(VI) : 0.27 $SeCN^-$: 0.19	0.25 - 10	[(b)]**Sea Water / [(b)] Rain Water:** Se(IV) : 120.8 / 102.7 Se(VI) : 99.5 / 100.9 $SeCN^-$: 69.4 / 93.6	(Wallschläger and London, 2004)
SPME-AEC-ICP-DRC-MS	^{78}Se(IV) ^{78}Se(VI)	Se(IV) : 2.2 Se(VI) : 1.9	10 - 20,000	[(b)]**River Water:** Se(IV) : 95.3 - 106.5 Se(VI) : 98.3 - 106.2	(Tsoi and Leung, 2011)
CE-HG-AFS	Se(IV) Se(VI)	Se(IV) : 25,000 Se(VI) : 33,000	-	[(b)]**River Water:** Se(IV) : 84 - 114 Se(VI) : 84 - 114	(Lu and Yan, 2005)
CE-ICP-MS	^{82}Se(IV) ^{82}Se(VI) ^{82}SeMet $^{82}SeCys_2$ Me^{82}SeCys	Se(IV) : 2,310 Se(VI) : 2,230 SeMet : 1,480 $SeCys_2$: 1,270 MeSeCys : 1,330	10,000 - 400,000	[(b)]**Groundwater:** Se(IV) : 97.5 Se(VI) : 98.3 SeMet : 99.8 $SeCys_2$: 88.9 MeSeCys : 96.2	(Liu *et al.*, 2014)

[(a)]species spiked individually, [(b)]species spiked simultaneously, [(c)] isotopes noted for atomic MS methods, total ion chromatogram used for GC-MS

Unlike the fractionation methods discussed above, separation methods (usually) do not include a "total Se" fraction, and therefore often do not necessarily account for all of the Se in a given sample. In some cases, one or more species may not interact with the chromatographic column and therefore elute in the void volume – this is frequently encountered in liquid chromatography and is discussed in more detail, below. Conversely, certain Se species in a sample may simply never enter the column, as is the

case for GC methods examining volatile species or those relying on (typically species-specific) derivatization protocols. For this reason, analysts often combine speciation methods with total Se determinations allowing for a comparison of the "sum of species" with the total Se value; the difference between these two measurements has varying analytical implication, depending on the speciation method employed.

Prior to the speciation analysis of a water sample using a separation method, it is not uncommon for one of the sample preparation methods discussed above (in the context of fractionation) to be employed for preconcentration or sample cleanup. Water samples – particularly those collected from contaminated industrial sources – often contain high concentrations of other chemical components, which often interfere with the separation methods used for analysis of Se species. These pre-treatment protocols, and their applicability to the various analytical procedures, are discussed in more detail below.

4.1 Gas Chromatography

As noted above, some volatile Se species are formed through biological activity or photochemical reactions in natural waters, making them ideal candidates for identification and/or quantification by GC. When a gas or liquid sample is injected in a gas chromatograph it is heated and passed through a chromatography column which is either hollow or packed with an inert material coated with a thin layer of non-volatile liquid. The column is heated (sometimes following a gradient of increasing temperature) and analytes are separated based on their volatility as a carrier gas (mobile phase) is passed through the column. Due to the fact that the eluent is in the gas phase, a wide variety of detectors can be coupled with GC instruments. Among the most popular is the flame ionization detector (FID) due to its nearly universal nature: the sample is burned in a H_2 flame generating a signal for most carbon-containing species. Mass spectrometry (MS) is another popular detection method, with several options available.

Most widely applied to GC is an instrument employing electron ionization (EI), which is a high energy ionization method that causes an analyte molecule to fragment in a characteristic pattern, often allowing for its identification, particularly when compared to a library of mass spectra. Chemical ionization (CI) is a softer ionization technique which keeps a larger proportion of the analyte molecules intact for analysis. Both of these ion sources can be associated with either a single or triple quadrupole mass analyzer – the former takes a single mass spectrum, while the latter allows for further selectivity and targeted fragmentation of an analyte molecule or fragments thereof. The significant advantage of using this type of MS system is the ability to identify analytes based on their mass spectra. ICP-MS instruments can also be modified to analyze GC eluent for element-specific detection.

The incompatibility of water with GC means that direct injection of a natural water sample is to be avoided, so the analysis of volatile species generally includes an extraction step, either directly from the water (Lenz *et al.*, 2008a; Lenz *et al.*, 2011) or from the sample's headspace (Ghasemi *et al.*, 2010; Ghasemi and Farahani, 2012). For example, Lenz *et al.* (2008a; 2011) exposed their sample to a carboxen/ polydimethylsiloxane SPME fiber to collect and concentrate DMSe and DMDSe from water samples. The Se was then eluted and injected for GC-MS analysis. Limits of quantification for this method were noted to be 1.5 µg/L and 2.5 µg/L (as Se) for DMSe and DMDSe, respectively. A similar method used nano-structured lead dioxide (deposited on a platinum wire) as the solid phase in a headspace-SPME protocol prior to thermal desorption and GC-MS, but achieved significantly lower detection limits (16 ng/L and 11 ng/L for DMSe and DMDSe, respectively, see Table 3), potentially due to the high efficiency of nanomaterials for analyte extraction. This method was used for the analysis of several environmental water samples; there, the only sample shown to contain native concentrations of DMSe and DMDse was the wastewater, which contained 4.3 and 1.9 µg/L, respectively (Ghasemi and Farahani, 2012). The authors do not comment on the source of the wastewater, so it is unknown whether biological activity played a role in volatilization of Se.

4.2 Derivatization Approaches

Since the majority of Se species are not naturally volatile, their analysis by GC requires sample preparation involving the derivatization of these non-volatile species into appropriate analytes. Probably the most common derivatization procedure allowing for the analysis of inorganic Se by GC involves the production of piazselenols. These volatile species form through the reaction of Se(IV) with phenylenediamines under acidic conditions and are then extracted into an organic solvent for subsequent analysis (Najafi *et al.*, 2012). This liquid-liquid extraction step is particularly beneficial for the analysis of Se in environmental samples due to its preconcentration capabilities. Enrichment factors between 112 (dispersive liquid-liquid microextraction, DLLME (Bidari *et al.*, 2008)) and 2491 (ultrasound-assisted emulsification microextraction, USAEME (Najafi *et al.*, 2012)) have been achieved during the analysis of inorganic Se in water samples. Similar to the fractionation methods discussed above, only one species is sensitive to this derivatization, meaning that the others (in this example Se(VI) and/or total Se) are calculated based on the difference between measurements after conversion to Se(IV).

Derivatization protocols have also been employed to analyze non-volatile organic Se species. These methods have been used for the analysis of SeMet extracted from yeast and derivatized with an alkyl chloroformate (Mester *et al.*, 2006; Ouerdane and Mester, 2008; Yang *et al.*, 2004a) or cyanogen bromide (Yang *et al.*, 2004b), as well as SeMet extracted from algae and derivatized by silylation (Fan *et al.*, 1997; Fan *et al.*, 1998); this silylation-GC-MS procedure was also demonstrated to be effective for MeSeCys and SeCys in a standard solution. With a thorough extraction and preconcentration protocol, these methods could theoretically be applied to organic Se species in waters, though to the best of our knowledge this has not yet been attempted.

An important benefit of the use of GC for separation of derivatized Se before detection (by FID, MS, etc.) is the ability to account for any potentially volatilizing Se species that may be present in the native

546 sample, if they are also extracted into the organic phase. This will not necessarily be a quantitative
547 recovery – one of the GC methods discussed above should be used for such purposes – but ensures that
548 the target species are not overestimated due to the presence of volatile Se, as is a difficulty in methods
549 such as SSHG, as discussed above.

550 *4.3 High Performance Liquid Chromatography*

551 High performance liquid chromatography (HPLC) is easily the most widely used method for Se speciation
552 analysis, mainly due to the fact that it encompasses a broad range of analytical methods, all of which
553 operate following a similar set of principles. For the purposes of this review, any methods employing a
554 solid stationary phase and liquid mobile phase will be discussed under the HPLC heading, with different
555 stationary phase types being considered in more detail separately, with the exception of size exclusion
556 chromatography, which is discussed in Section 4.5 with the other size-based speciation methods.

557 As already discussed extensively, the major Se species in natural waters are present as oxyanions under
558 ambient conditions; therefore, it follows that the chromatographic method most popular for the
559 analysis of these waters is anion exchange chromatography (AEC). Here, the column (stationary phase) is
560 composed of cationic exchange sites – commonly quaternary ammonium groups on cross-linked
561 styrene-divinylbenzene copolymer – and the mobile phase (eluent) is a salt solution, sometimes of high
562 pH (Harris, 2007; Skoog *et al.*, 2007).

563 It is common for commercially available ion chromatography systems to come equipped with a
564 conductivity detector (CD), allowing for the analysis of relatively high concentrations of the major anions
565 in water samples. Due to the high conductivity of the mobile phase used in these systems, they typically
566 employ an eluent suppressor which itself is a small-scale ion exchange membrane which uses an electric
567 potential and/or regeneration solution (H_2SO_4 is common in AEC) to convert the highly conductive ions
568 in the mobile phase (generally OH^- for AEC or H^+ for cation exchange chromatography (CEC)) into water.

This allows the analytes' conductivity to be detected without interference from the mobile phase (Small *et al.*, 1975). Conductivity detection combined with AEC has been applied to Se speciation analysis. For example, Lenz *et al.* (2006) achieved detection limits of 25 µg/L for Se(IV) and 11 µg/L for Se(VI) using suppressed AEC with a sodium hydroxide eluent for Se speciation in an aqueous extract of anaerobic granular sludge. While detection limits in this range are quite high in comparison to others discussed here and too high for the analysis of natural waters, they are applicable to some industrial samples. As Lenz and coworkers (2006) highlighted, these values are well below the allowable total Se concentration of 1 mg/L for liquid effluents in metal finishing industries in much of Europe at the time of their publication. Based on this, the cost-effectiveness of an AEC-CD system, and the minimal sample preparation required for typical industrial water samples, this is an ideal method to perform inorganic Se speciation analysis in industrial settings.

Similar instrumental setups have been coupled to more sensitive detection methods – most commonly ICP-MS. While the mobile phase is not as problematic here as it is with CD, the use of a membrane suppressor is still beneficial for inorganic Se speciation analysis. The use of a sodium/potassium hydroxide elution gradient (as is common with AEC) does cause signal suppression in ICP-MS. Through the application of eluent suppression, Wallschläger and Roehl (2001) observed a 1.5-, 1.4-, and 1.3-fold increase in the signal-to-noise ratio on ^{82}Se for Se(IV), Se(VI), and $SeCN^-$, respectively (note that those species elute in that order using an increasing concentration sodium hydroxide elution gradient). In addition to reducing analysis sensitivity, the introduction of large quantities of sodium hydroxide (or similar salt solutions) into an ICP-MS decreases the lifetime of the instrument and its components – particularly the cones and lens stack which require more frequent cleaning.

Whereas eluent suppression in AEC can be extremely useful, it cannot be universally applied to Se speciation analysis. Samples containing, or potentially containing, organic Se cannot be analyzed

because many of these species are incompatible with membrane suppressors. SeMet, for example, is zwitterionic at neutral pH, causing it to be retained on the cationic membrane during the pH change associated with the suppression process (Wallschläger and Roehl, 2001). The amino acid functional groups on other organic Se species cause similar difficulties. Regardless, low detection limits can be achieved by directly coupling AEC to ICP-MS, even without eluent suppression, particularly when other minor adjustments are made to increase sensitivity. For example, in one scenario, the use of a dynamic reaction cell (DRC), to eliminate the $^{40}Ar_2^+$ interference and allow for the monitoring of ^{80}Se, and the addition of 2% methanol to the eluent to increase the mass spectra signal, allowed for instrumental detection limits of 5 ng/L to be obtained for SeMet, Se(IV), and Se(VI) (LeBlanc *et al.*, 2016).

The use of HG as an interface between an HPLC column and ICP-source instrument (MS or OES) can help improve instrumental performance. Unlike with conventional spray chambers which are used for the direct coupling of AEC to an ICP instrument, the sample introduction efficiency of volatile hydrides is quite high, and maintaining a "dry" plasma increases Se ionization efficiency, which is relatively low compared to other elements (Darrouzès *et al.*, 2008; Wallschläger and London, 2004). As seen in Table 3, Wallschläger and London's (2004) AEC-HG-ICP-DRC-MS method obtained sub-ng/L detection limits for Se(IV), Se(VI), and $SeCN^-$. A similar study, also employing collision cell (CC) ICP-MS, noted detection limits of 2 and 5 ng/L for Se(IV) and Se(VI) (compared to Wallschläger and London's (2004) 0.15 and 0.27 ng/L), but here the authors also examined the organic species SeMet and $SeCys_2$, achieving detection limits of 8 and 6 ng/L, respectively. However, the recoveries of standards spiked into real sample matrices were not investigated (Darrouzès *et al.*, 2008).

The combination of HG and AEC requires fast and efficient online conversion of all species of interest to volatile hydrides, which can be a challenging task, particularly for organic Se species. One such setup was devised by Darrouzès *et al.* (2008) and involved effluent from the AEC column mixing with the

615 potassium iodide / sodium hydroxide reductant before flowing through a UV-transparent piece of tubing
616 wrapped around a UV lamp; the length of this tubing corresponded to an irradiation time of 60 seconds.
617 The mixture then was further mixed with hydrochloric acid and borohydride to form volatile hydrides,
618 passed through a gas-liquid separator and into the ICP-MS. One major disadvantage of such a post-
619 column HG setup is the potential for peak broadening during the flow-through. However, this is
620 unavoidable if quantitative conversion to volatile hydrides is to be achieved, and therefore the
621 researcher must be diligent in their optimization of the employed chromatographic method to ensure
622 adequate separation between each Se species.

623 As with all separation methods, sensitivity can be gained by sample treatment prior to analysis. SPME is
624 a popular technique to employ before chromatographic analysis as it allows preconcentration of the
625 analytes and/or removal of interfering components in the sample. An example of detection limits
626 obtained from such a process can be found in Table 3, which makes note of a procedure involving
627 surface-modified activated carbon for the selective preconcentration of Se(IV) and Se(VI) prior to HPLC-
628 ICP-DRC-MS (Tsoi and Leung, 2011). The application of different solid phases in SPME can result in a
629 more thorough sample pretreatment, as has been demonstrated by Bueno and Potin-Gautier (2002)
630 who used Amberlite IRA-743 for the preconcentration of SeMet, $SeCys_2$, Se(IV), and Se(VI) before AEC-
631 ICP-DRC-MS analysis. One difficulty that arose during the optimization of this procedure was due to the
632 fact that the chosen resin was not Se-selective and therefore interference from competing ions in
633 solution caused lowered retention efficiencies for SeMet in ground waters and freshwaters – SeMet was
634 not retained on the resin for any of the natural water samples, though 64% recovery was noted for Milli-
635 Q water. However, recovery of the other three species in natural waters was 92 – 97%.

636 Removing interfering ions in a sample is often equally or more important actually than preconcentrating
637 the analyte, particularly in the analysis of waters collected from industrial settings. Sulphate, for

example, is commonly found at elevated concentrations in waters containing appreciable amounts of Se and causes retention time shifts during AEC (Wallschläger and London, 2004). These shifts can be accounted for by spiking experiments in some samples, but cause more significant difficulties in others, especially those containing weakly-retained organic Se which has been noted to co-elute in the void volume of samples containing even relatively low concentrations of sulphate (LeBlanc *et al.*, 2016; Wallschläger and London, 2004). Simple pretreatment procedures to remove suphate by precipitation with barium have proven effective for natural (albeit contaminated) water samples (LeBlanc *et al.*, 2012; LeBlanc *et al.*, 2016), while industrial samples required more thorough cleanup protocols combined with preconcentration for the analysis of organic Se species by AEC (LeBlanc *et al.*, 2016).

Due to the ubiquitous nature of the Se oxyanions in environmental water samples, AEC is by far the most common chromatographic method used for their analysis. Other column types are frequently used for Se speciation in biological matrices, as the species of interest are typically organic – for example, Se speciation is yeast is a fairly common research endeavour (Bierla *et al.*, 2012; McSheehy *et al.*, 2005; McSheehy *et al.*, 2006; Mester *et al.*, 2006). However, on a reversed phase column, for example, the majority of the Se present in most water samples (Se(IV) and Se(VI)) (co)elutes in or near the void volume, which is not ideal when the goal is to simultaneously analyze all Se present in a given sample. It is possible to avoid this coelution through the use of an ion pairing agent such as tetrabutylammonium hydroxide (TBAH) which complexes with Se(IV) and Se(VI) allowing them to be analyzed by reversed phase chromatography. The late Joseph Caruso's research group, for example, has optimized an ion-pairing reversed phase (IPRP) chromatographic method for the simultaneous analysis of inorganic and organic Se species in aqueous samples (Afton *et al.*, 2008).

The application of a species-specific calibration is vital to precise and accurate speciation analyses due to the potential for varying instrumental response to different Se species under different conditions. While

this holds true for many types of analysis, it is particularly important for chromatographic methods involving gradient elutions where the matrix in which the analyte is contained is continuously changing. This can cause changes in the relative response of the instrument to the analytes present, depending on their elution time; for example, increased methanol concentrations (to a certain extent) cause enhanced Se signals in ICP-MS (Larsen and Stürup, 1994). This can elicit difficulties when attempting to quantify species for which no standards are available – potentially because the species has yet to be identified – as is sometimes the case in environmental samples, particularly collected from industrial settings (LeBlanc and Wallschläger, 2016; Petrov *et al.*, 2012). In scenarios where it is desired to quantify species for which standards are not available or whose identities are unknown, a relative response curve can be generated by adding the analyte to the internal standard solution being mixed with the eluent via a post-column "T" connection and running the chromatographic gradient without a sample injection. This allows for the eluent-induced variation in instrumental response to be quantified and accounted for in subsequent calculations of species concentrations (Amayo *et al.*, 2011).

So far, our discussion of HPLC methods has been limited to detectors which do not provide structural information (i.e. CD and ICP-MS), meaning species identification is based only on retention time matching between samples and standards. While this is sufficient for some samples, it is not always adequate for those containing unknown species (which may elute at similar times to other species in the analysis) or concentrations of interfering ions high enough to cause retention time shifts. The use of electrospray ionization tandem mass spectrometry (ESI-MS/MS) satisfies the need for structural information as the soft ionization technique typically allows the molecular ion to be observed, and fragmentation by collision induced dissociation (CID) generates further structural information. This method is commonly applied to biological samples but less frequently to water samples, though it has been shown to be effective in water samples containing organic Se (Afton *et al.*, 2008; LeBlanc *et al.*, 2016).

4.4 Capillary Electrophoresis

Capillary electrophoresis (CE) involves the migration of ions through a capillary to which an electric field is applied. A major advantage of CE is its high resolving power due to the elimination of two terms in the van Deemter equation for calculating theoretical plate height: the term for multiple paths is removed because the capillary is open (opposed to a packed column), and the mass transfer term is eliminated since CE involves no stationary phase (Harris, 2007; Skoog *et al.*, 2007); both factors differentiating this method from HPLC.

Due to the extremely small diameter of the capillary, both injection volume and flow rate are very low for CE analyses, and it follows that detection limits are relatively high for any water samples analyzed directly. Therefore, sample preparation methods employing preconcentration are often employed. In fact, HFLPME was developed as a sample preparation technique for CE (Pedersen-Bjergaard, 1999). The use of hollow fibres is ideal due to the low volumes of extracting solution involved, but other techniques have proven to be also very effective. For example, Yan and coworkers (2015) were able to decrease detection limits for the analysis of Se(IV), Se(VI), SeMet, and $SeCys_2$ by more than an order of magnitude by employing an SPE step prior to CE-ETV-AAS. Similarly, Duan *et al.* (2012) used SPE-CE-ICP-MS to obtain detection limits of 57 and 71 ng/L for Se(IV) and Se(VI), respectively, which are on the order of those obtained by single quadrupole ICP-MS coupled to other separation methods. These low detection limits were based on extremely efficient preconcentrations where enrichment factors of 41,367 and 61,935 were obtained for Se(IV) and Se(VI), respectively. These sample pretreatment methods also help to overcome the other issue caused by the small injection volumes associated with CE: inhomogeneities due to nanolitre-sized subsample selection. This can be overcome by concentrating the analyte(s) from a larger volume of sample into an injectable size through one of the methods discussed above (Michalke, 2005).

As with other separation methods, a wide variety of detectors are available, but coupling them with CE provides a unique set of challenge due to the low flow rates and the fact that there is a charge on the capillary. While UV/Vis spectrophotometers make simple detectors, their limited sensitivity combined with the low injection volume provides relatively high detection limits (Sladkov *et al.*, 2003). Atomic mass spectrometry is generally a much more sensitive detection method, but interfacing a CE system with an ICP-MS is often a major difficulty that must be overcome to achieve these desired low detection limits. One of the main reasons this is such a difficult endeavour is the discrepancy between flow rates employed in CE and those required to obtain efficient nebulization for ICP-MS. Sometimes, a make-up solution is used to increase the flow rate (Michalke, 2005), but this has the disadvantage of diluting samples and thus increasing detection limits. Liu *et al.* (2014) combated the issue of interfacing these systems through the use of an ESI-MS sprayer, the tip of which was inserted directly into a low-volume spraychamber. The ESI sprayer was designed to operate at low flow rates and be held at a potential, making it an ideal interface. Without any sample preconcentration, detection limits in the range of 1.3 µg/L to 2.3 µg/L were obtained for all Se species, following this method (see Table 3).

4.5. Size-Based Speciation Methods

In Part 1 of this review (Kumkrong *et al.*, 2018), we discussed the different pools of Se commonly examined during total Se analyses – particularly, the difference between "total recoverable" and "dissolved" fractions. This type of distinction gives a preliminary indication of the Se size fractions present in a water sample, where the total recoverable fraction can include particulate Se or that which is adsorbed to organic matter or suspended minerals, or present in or on the surface of microorganisms in the water column (Schlekat *et al.*, 2000; Simmons and Wallschläger, 2005). Suspended Se can be separated from the bulk water (and dissolved Se species) through filtration or centrifugation and subsequently analyzed in more detail. Methods such as scanning electron microscopy-energy disperse X-

731 ray spectroscopy (SEM-EDXS) and transmission electron microscopy (TEM) allow researchers to examine
732 the size and crystallinity of Se^0 (nano)particles collected on filters or by centrifugation (Jain *et al.*, 2015;
733 Jain *et al.*, 2016). Similar experiments have been conducted to examine Se^0 nanoparticular size and
734 distribution within the biomass of various organisms (Andrews *et al.*, 2009).

735 Size-exclusion chromatography (SEC) has been used extensively for the study of Se in biological
736 materials, particularly in metabolomics studies (Arnaudguilhem *et al.*, 2012; Bierla *et al.*, 2012; Gilbert-
737 López *et al.*, 2017; McSheehy *et al.*, 2002; Oliveira *et al.*, 2016). Like HPLC, this method involves the use
738 of a solid stationary phase and liquid mobile phase. Separation is based on the ability of analytes to
739 travel in and out of pores in the stationary phase – smaller molecules spend more time in these pores
740 and thus elute later. SEC is commonly employed as a preparatory step for samples containing a large
741 number of analytes spanning a wide range of sizes; fractions are collected and analyed further using a
742 higher resolution separation method, frequently coupled to a molecular mass spectrometer for
743 structural identification. SEC is not commonly used for analysis of Se in environmental waters, but has
744 been employed for the examination of dissolved organic matter-bound Se (Martin *et al.*, 2017; McKenzie
745 and Young, 2013).

746 **5. The Importance of Selenium Speciation in Biological Remediation Processes**

747 The interaction between Se and microorganisms has been investigated for several decades, with
748 significant amounts of research being focussed on bacteria and yeast. From an environmental viewpoint
749 there has been particular interest in the use of microorganisms in the treatment of Se-contaminated
750 waters. Here, we provide some brief background on these processes with an emphasis on the role of
751 microorganisms in the alteration of Se speciation in waters, as well as the importance of Se speciation in
752 the remediation processes. For a much more extensive review of the biological treatment processes

753 used for Se remediation, we refer the reader to reviews by Tan and coworkers (2016), Lenz and Lens
754 (2009), Wu (2004), and Dungan and Frankenberger (1999).

755 Physio-chemical methods for Se remediation often involve adsorption of Se onto various materials
756 including amorphous iron oxyhydroxide, manganese dioxide (Balistrieri and Chao, 1990), granular iron,
757 and organic carbon (Gibson *et al.*, 2012). In most cases, adsorption of Se(IV) is stronger (sometimes
758 significantly) than that of Se(VI). Additionally, Se contamination often occurs simultaneously with
759 elevated concentrations of inorganic salts. These salts, SO_4^{2-} in particular, cause chemical and physical Se
760 removal methods to become difficult and often prohibitively expensive (Dungan and Frankenberger,
761 1999), making biological treatment a viable option in many systems. Generally, biological removal of Se
762 oxyanions from water occurs through three mechanisms: (a) dissimilatory reduction to insoluble Se^0, (b)
763 assimilatory reduction and incorporation in amino acids and sometimes Se-containing proteins, and (c)
764 reduction and methylation to volatile Se species (Dungan and Frankenberger, 1999; Losi and
765 Frankenberger, 1997). While (a) is a mostly distinct process resulting in the sequestration of Se^0 in
766 sediments (Oremland *et al.*, 1989), (b) and (c) are quite similar, with the difference being the final fate of
767 the Se; Se-containing proteins and (some) amino acids remain with the organism's biomass, while
768 volatile species such as DMSe and DMDSe are released to the atmosphere and typically removed from
769 the aquatic system (Wu, 2004).

770 It is generally accepted that bacterial bioreduction of Se(IV) occurs more readily than that of Se(VI),
771 presumably due to the observation that a relatively larger number of microorganisms have the ability to
772 reduce Se(IV), compared to Se(VI) (Dungan and Frankenberger, 1999; Losi and Frankenberger, 1997).
773 Since Se(VI) is present in most Se-contaminated waters in appreciable amounts, this is important to take
774 into consideration when selecting a bacterial strain for biological remediation. Additionally, biological
775 reduction of Se(VI) and Se(VI) is highly sensitive to surrounding environmental conditions, particularly

776 sulphite, due to competitive uptake inhibition (Lortie *et al.*, 1992), and nitrate, the presence of which
777 appears required for complete reduction of Se(VI) to Se^0 by some bacteria (Dungan and Frankenberger,
778 1999). Large-scale biological reactors typically consist of multiple vessels which contain bacterial
779 colonies on various substrates; some systems rely on the addition of a carbon source to act as an
780 electron donor for the reduction process. A limitation of bacterial Se-reduction systems is the toxicity of
781 Se to many types of bacteria, which has been observed in several bacteria-based treatment systems
782 (Jain *et al.*, 2016; Mal *et al.*, 2017; Zehr and Oremland, 1987). Based on these observations, systems
783 intending to treat (waste)waters containing very large concentrations of Se should employ highly Se-
784 tolerant bacteria to ensure optimal efficiency (Lenz and Lens, 2009; Macy and Lawson, 1993). The Se^0
785 produced by bacteria is frequently of nanoparticulate size, ranging from about 50 to 500 nm in diameter
786 (Buchs *et al.*, 2013; Lenz *et al.*, 2009). Due to the ability of nanoparticles of this size to remain suspended
787 in aqueous environments for extended periods of time (Lenz *et al.*, 2008a), measures must be taken to
788 promote adequate settling before any effluents are discharged to ensure Se concentrations are below
789 regulatory limits. Buchs and coworkers (2013) examined the stability of nanoparticular biogenic Se in the
790 context of bioremediation effectiveness, demonstrating that dissolved organic matter serves to increase
791 colloidal stability, but the addition of counter-cations induced fast settling of the nano-Se. With this in
792 mind, understanding the size distribution of Se suspended an effluent could help determine the final
793 steps required for its treatment.

794 Methylation, and subsequently volatilization of Se by various biota, has been postulated to be a
795 detoxification mechanism which occurs when exposed to elevated levels of inorganic Se (Dungan and
796 Frankenberger, 1999; Wu, 2004). This process has been observed in bacteria (McCarty *et al.*, 1993; Rael
797 and Frankenberger, 1996; Ranjard *et al.*, 2003; Weres *et al.*, 1989), as well as in algae (Fan *et al.*, 1997;
798 Neumann *et al.*, 2003) and plants (Pilon-Smits and Quinn, 2010). The incorporation of algae and other
799 plants into biological treatment systems has been of particular interest recently.

800 Constructed wetland systems have been employed for the bioremediation of Se-contaminated waters.
801 These systems employ both reduction (to Se^0 followed by sequestration in the sediments) and
802 volatilization to remove Se from the water. In these wetland systems, it is often the interactions
803 between components that provide their efficiency at Se removal. For example, in a series of constructed
804 wetlands, those containing rabbitfoot grass volatilized Se significantly more efficiently than wetlands
805 with other plant species (the study also examined cattail, Baltic rush, smooth cordgrass, saltgrass, tule,
806 widgeon grass, and saltmarsh bulrush). The authors postulated that this was related to the microbes
807 associated with the rabbitfood grass, which aid in the conversion of Se(VI) to organic Se species (Lin and
808 Terry, 2003), which is the limiting step in volatilization by plants (Pilon-Smits and Quinn, 2010). A similar
809 effect was observed in the volatilization of Se from an alfalfa hay mesocosm: a significantly larger
810 fraction of Se was volatilized when Se was supplied as SeMet rather than as Se(IV), which was still
811 significantly larger than when supplied as Se(VI) (Huang *et al.*, 2012b). Consideration of these rate-
812 limiting processes is important when developing these systems to ensure maximum Se removal
813 efficiency.

814 Generally, it is the initial and final concentrations (and speciation) of Se in bioremediation systems that
815 are thought of as being the most important; however, the intermediate processes involved need to be
816 considered for a more thorough understanding of the potential eventual fate of Se in these systems. For
817 example, Se(VI) taken up by algae and cattails transformed in a simulated wetland microcosm was
818 converted to volatile Se species, with SeMet being a step along this metabolic pathway. After the plants
819 were harvested, SeMet was measured in the biomass (by extended X-ray absorption fine structure
820 spectroscopy) (Huang *et al.*, 2013). In such a scenario, there is the potential for this SeMet to be
821 released to the water upon the death of the plant – as has been seen for algae (LeBlanc and
822 Wallschläger, 2016). Likely, this highly bioavailable species would be taken up again by other biota in the
823 constructed wetland, but it is possible that it could be released with the effluent, increasing its

824 bioavailability over an effluent containing only inorganic Se. This scenario has been observed previously,
825 where following biological treatment, the effluent from the system was more bioavailable than the
826 influent, despite a decrease the total Se concentration (Amweg *et al.*, 2003).

827 Unfortunately, very few studies actually examine the discrete speciation of Se in the effluent, though
828 some do monitor for an org-Se fraction, and discrepancies between total dissolved Se and the measured
829 Se(IV), Se(IV), and Se^0 have been noted (Hansen *et al.*, 1998; Lenz *et al.*, 2008b). Table 4 provides several
830 examples of Se concentrations (and speciation data, where available) in contaminated waters before
831 and after treatment. Based on what is known about the potential for the production of organic Se
832 species in biological treatment systems, monitoring for their presence could be extremely beneficial in
833 predicating the toxicity of the effluent. Sensitive and robust analytical methods for the measurement of
834 Se speciation in water samples are required for such an endeavour, as environmentally-relevant
835 concentrations of org-Se may be only a small fraction total Se in a given sample.

836

837 **Table 4:** A non-exhaustive list of Se concentrations in various waters before and after treatment**.** Note that examples using synthetic
838 contaminated waters were intentionally excluded from this list

Site or Cause	Se Concentration Before Treatment (µg/L)	Se Concentration After Treatment* (µg/L)	Treatment Type**	Reference
Ambassador Duck Club, Inflow from the Jordan River, Utah, USA	TSe : 1 ± 0.6 Se(IV) : <5 - 16 % Se(VI) : 79 - 95 % org-Se : <5 - 15 %	TSe : 0.7 ± 0.2 Se(IV) : 0 % Se(VI) : 0 - 66 % org-Se : 34 - 100 %	Natural flow-through wetland system with 9 ponds; pondweed, spiral tasselweed vegetation	(Dicataldo *et al.*, 2010)
Imperial Wetlands, New River, California, USA	2.7 - 5.4	2.0 - 4.8	Constructed wetland; bulrush, tamarisk, wild grasses vegetation; 11000 m^3/day flow-through	(Johnson *et al.*, 2009)
Brawley Wetlands, New River, California, USA	2.2 - 3.9	1.1 - 2.0	Constructed wetland; bulrush, tamarisk, wild grasses vegetation; 2700 m^3/day flow-through	(Johnson *et al.*, 2009)
Treated Refinery Water (USA)	n/a	43	Unknown	(Siddique *et al.*, 2007)
Agricultural Drainage Water (USA)	Se(VI) : 338	Se(IV) : 6.3 µg/L Se(VI) : 32.9 µg/L Org-Se : 14.3 µg/L	Laboratory-scale *Citrobacter braakii* colonies enhanced with Zero-Valent Iron (ZVI), 0.1% molasses added as a carbon source	(Zhang and Frankenberger, 2006)
Corcoran, California, USA	16 ± 2	6 - 14	Constructed wetland with 10 flow-through "wetland cells"; cattail, Baltic rush, smooth cordgrass, saltgrass, tule, widgeon grass, saltmarsh bulrush, rabbitfoot grass vegetation	(Lin and Terry, 2003)
Unnamed Wetland, Mouth of the San Pablo Bay, California, USA	20 – 30 Se(IV) : 51 - 101 %	< 5 Se(IV) : 55 - 87 %	Constructed wetland, 3 surface-flow sections, 6400 m^3/day flow-through Section 1: dense vegetation (cattails, saltmarsh bulrush) Section 2: mostly open water (10-15% coverage of cattails, saltmarsh bulrush, tules) Section 3: mid-density vegetation (cattails, saltmarsh bulrush)	(Hansen *et al.*, 1998)
Panoche Water District Drainage Water	Se(IV) + Se(VI) : 289 ± 90.4 Se(IV) : < 5 %	< 12 ± 9 < 5 on 47 % of sampling days	Pilot-scale biological reactor, *Thauera selenatis*; acetate (as acetic acid) added as a carbon source	(Cantafio *et al.*, 1996)

839 *Total Se unless otherwise stated

840 **For studies discussing several treatment methods, only the method the authors found most effective is listed here

6. Selenium Analysis in Environmental Samples – Future Outlook

In Sections 3 and 4, wherever possible, we focussed on examples discussing the speciation analysis of Se in environmental samples – mostly waters and wastewaters. Detection limits of these methods can span several orders of magnitude depending on a number of factors including detector type, preconcentration efficiency, and injection volume. Contrasting this to the methods and associated detection limits noted in Part 1 of this review (Kumkrong *et al.*, 2018) – which discussed environmental regulations and standard methods of analysis used to establish compliance – emphasizes the disconnect between actual and potentially concerning environmental conditions and the way in which those conditions are routinely monitored. In many scenarios, a measurement of the total Se in a water sample is not adequate to accurately predict the potential for toxic effects in most aquatic environments. As noted previously, the U.S. EPA has begun to address this through the application of (fish) tissue-based water quality criteria for Se, which accounts for differences in the bioavailability between Se species (U.S. EPA., 2016). Canada intends on taking the same analytical approach in new regulations currently being developed. However, this type of analysis cannot be applied to waters that do not act as habitats for fish and other wildlife – for example, the bioavailability of Se can vary widely between different types of effluents. In such a scenario, a direct analysis of the effluent is required, and knowledge of whether it contains Se in oxidized, reduced, inorganic, or organic forms, is vital to accurate predictions of its eventual toxicity once it is released into natural waterways. In Section 5, which focussed on (biological) remediation techniques for treating Se-contaminated waters, we discussed the importance of Se speciation in the effectiveness of these methods. An analysis of Se speciation in incoming waters helps ensure appropriate techniques are implemented to ensure adequate removal efficiency. Se speciation analysis of the effluent from these systems can demonstrate how the processes involved alter Se speciation – this data may also be important for predicting the ability of Se to enter the food chain of the receiving environment.

As noted throughout this two part review, methods are available to perform the types of analyses discussed above, though some are prohibitively expensive for applications in regular monitoring efforts. However, research is still underway and new sensitive and cost-effective analysis methods are continually emerging.

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