Review of Bioavailability and Toxicity of Nickel in Alloys

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ToxStrategies

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Acronyms

ABC	airborne benchmark concentration
ALF	artificial lysosomal fluid
CLP	Classification, Labeling and Packaging of Substances and Mixtures (Regulation)
EPA	US Environmental Protection Agency
HERAG	Health Risk Assessment Guidance for Metals
IARC	International Association for Research on Cancer
LOAEC	lowest-observed-adverse-effect concentration
NOAEC	no-observed-adverse-effect concentration
NTP	National Toxicology Program
NTP	National Toxicology Program
ODEQ	Oregon Department of Environmental Quality
OEHHA	Office of Environmental Health Hazard Assessment (California)
SMR	standardized mortality ratio
SPSF	standard project submission form (OECD)
UN	United Nations

Executive Summary

ToxStrategies, Inc., reviewed the toxicokinetic, toxicological, and epidemiological literature to assess the potential for nickel in the form of an alloy to pose an inhalation cancer hazard and to determine whether it is appropriate to assess the potential health risks of nickel in alloy using the Oregon Department of Environmental Quality's (ODEQ's) airborne benchmark concentration (ABC) for insoluble nickel. This is an important issue, because when environmental samples of nickel in soil and air are assessed, the results are typically provided as "total nickel" concentrations, and the chemical form (or species) of nickel is not evaluated. Typically, total nickel is then assessed for potential hazards based on the toxicity of a specific nickel compound (e.g., nickel subsulfide) or compound group (e.g., soluble nickel). While this is not an uncommon practice in environmental risk assessment, it has the potential to be highly inaccurate when assessing nickel, because the potential for hazard varies considerably based on the the chemical and physical form of the nickel particles.

Nickel in an alloy form should be considered distinctly from "insoluble nickel," because nickel in alloys is generally not bioavailable, meaning that nickel ions are not readily released from the alloy into biological fluids. When nickel in an alloy is assessed using the groupings developed based on solubility alone, the potential for human health effects can be significantly overestimated.

In Oregon, two separate ABCs were set for nickel based on categorization as Group 1 nickel compounds ("insoluble") and Group 2 nickel compounds ("soluble") (ATSAC, 2015). However, the forms of nickel that are the basis for the "insoluble nickel" ABC are bioavailable, and are not insoluble under some biological conditions. Thus, it is very important to distinguish "insoluble nickel" from nickel in alloy form. In their April 2015 meeting, the Air Toxics Science Advisory Committee proposed to add nickel subsulfide, nickel oxide, and nickel metal as separate entities, based on the independent listing of these nickel compounds used by the California Office of Environmental Health Hazard Assessment (OEHHA, 2004). However, none of these criteria are applicable to nickel in an alloy form. In fact, OEHHA specifically exempts nickel in the form of alloys from regulation under Proposition 65. OEHHA noted that "nickel alloys are distinct from nickel compounds and are not included in Proposition 65 listing of nickel compounds… A nickel alloy is a mixture of nickel with one or more other elements…" (OEHHA, 2004). Furthermore, the National Toxicology Program formally reviewed nickel alloy for inclusion in the report of carcinogens in 2000 and decided that nickel alloy should not be listed, indicating that "the human data are inadequate and the rodent cancer data are not sufficient for listing" (NTP, 2013).

As described in this report, nickel in the form of an alloy should not be assessed as insoluble nickel, which is the basis of the ODEQ ABC value, for the following reasons:

1. Nickel in alloy form has very low bioaccessibility[1] in the lung and thus has low potential for toxicity and carcinogenicity.
2. The animal toxicology data support that nickel in alloy form has low potential to cause toxicity or to be carcinogenic relative to nickel compounds.
3. Occupational epidemiologic studies of workers exposed to nickel alloys, without exposure to other forms of sulfidic nickel, do not support that exposure to nickel alloys increases the risk of lung cancer, even at extremely high concentrations compared to environmental exposure.
4. The nickel ion bioavailability model (Goodman et al., 2011) describes the basis for observed differences in nickel lung toxicity for different nickel compounds and provides a biological basis explaining why nickel in alloy form should not be evaluated with other insoluble nickel compounds.

Overall, several lines of evidence clearly and coherently support that assessing the potential hazard posed by nickel in alloy form using guidelines and toxicity criteria for "insoluble nickel" exaggerates the potential hazard.

1. Background

Nickel has several properties of hardness, malleability, ductility, and corrosion resistance, which make the metal very suitable for combining with other metals to create various metal alloys (ATSDR, 2005; IARC, 2012). Most nickel is used to make stainless steel; in 2007, it was reported that approximately 52% of primary nickel consumed in the United States was used for stainless and alloy steel production, and 34% in forming non-ferrous alloys and superalloys (ATSDR, 2005; IARC, 2012). The International Agency for Research on Cancer (IARC) indicated that several million workers worldwide are exposed to airborne fumes and dusts containing nickel (IARC, 2012).

In nickel-producing industries, workers are exposed predominantly to insoluble nickel, whereas in nickel-using industries, soluble nickel is the predominant exposure (IARC, 2012). However, these two categories of nickel-using industries do not adequately distinguish between the species of nickel with varying toxicological profiles. For example, while metallic, sulfidic, and oxidic nickel substances are all described as "insoluble"—they behave differently in the human body than do soluble nickel substances. Further, these categories do not capture a third category of industry wherein nickel is bound in alloys that are formed and shaped into products, and in many cases, these alloys are produced by others in the nickel-producing industries.

The ODEQ ABC for insoluble nickel is based on the carcinogenicity of "nickel refinery dust," as quantified using epidemiology data from several nickel refinery worker studies. Nickel refinery dust is a mixture of many nickel moieties, including nickel sulfate, nickel subsulfide, and nickel oxide, and the exact carcinogenic nickel forms in nickel refinery dust are not specifically known (EPA, 1987). In fact, the nickel content of nickel refinery

[1] Bioaccessibility is measured as the *in vitro* dissolution in synthetic biological fluids, which is a surrogate for the amount of a substance (e.g., metal ion) available for absorption.

dust is not specifically described and likely varies by refinery. For example, that from the Port Colborne, Canada, refinery was described by EPA (1987), citing work by Gilman and Ruckerbauer (1962), as 20% nickel sulfate, 59% nickel subsulfide, and 6.3% nickel oxide.

When environmental samples are analyzed for nickel, the results are reported in terms of total nickel, with all chemical species or forms combined, and the actual species are not typically described. Because nickel toxicity varies considerably by species, as discussed herein, it is important to understand the form(s) of nickel in the sample in order to accurately assess the potential for a hazard. This report assesses the appropriateness of using the ABC value for insoluble nickel to assess the potential hazards posed by nickel in alloy form, because Precision Castparts Corporate (PCC) Structurals, in Portland, produces parts using alloys enriched in nickel. Hence, it is assumed that nickel released from PCC Structurals is primarily in the form of an alloy.

2. Basis for Nickel Toxicity

Different forms of nickel demonstrate differences in toxicity and the potential to induce cancer. To appropriately evaluate the potential hazards resulting from exposure to different forms of nickel, the nickel species and form should be identified, and assessments should be performed specific to those species and the physical-chemical characteristics of the particles. Nickel can complex to form a wide variety of inorganic and organic substances; the toxicological profiles of inorganic nickel compounds and metallic nickel have been shown to differ depending on the route of exposure and endpoint of concern (ATSDR, 2005; European Union, 2008; Goodman et al., 2011; see also www.nipera.org). Available data for nickel compounds indicate that the bioavailability of nickel ions at the target site (e.g., tissue, organ, or nucleus) is the determining factor in producing adverse health effects. As such, the ability of a nickel substance to deliver nickel ions to target sites has been used as a predictor of toxicity, sensitization, and carcinogenicity (European Commission, 2008; Goodman et al., 2011; Henderson et al., 2012).

Until recent years, water solubility had been used to differentiate and predict toxicity for nickel compounds, serving as a surrogate for bioavailability data and leading many regulatory jurisdictions to assign toxicity weights based on two groups: soluble or insoluble. Assessing the solubility or insolubility in the acidic conditions of intestinal and gastric fluids (oral bioaccessibility) has been shown to be a robust method for assessing the potential bioavailable fraction and related oral toxicity of metals, including nickel (EPA, 2007c; Henderson et al., 2012; OECD, 2014). However, the oversimplified grouping into water soluble or water insoluble does not apply to bioavailability in the lung, which does not correspond directly to solubility.

Recently, and specific to nickel, the "nickel ion bioavailability model" was developed to provide a much more scientifically robust basis for evaluating toxicological and carcinogenic potential of nickel in the lung (Goodman et al., 2011). This model incorporates various factors based on data from *in vitro* and *in vivo* studies to determine

the bioavailability of the nickel ion at the cell nucleus, and as a result, predicts carcinogenic potential of nickel compounds. These factors include respiratory cellular toxicity (but not specifically carcinogenicity), clearance, retained dose, extracellular dissolution, intracellular uptake and dissolution, and delivery to and dissolution near the nucleus (see Goodman et al., 2011, for a full description). The nickel ion bioavailability model predicts that sulfidic and oxidic nickel compounds are associated with an increased risk of lung cancer, while water-soluble nickel compounds and nickel metal alone will not have this same potential. In accordance with the nickel ion bioavailability model, because nickel in alloy form is of very limited solubility in either acidic (lysosomal fluid) or neutral conditions (alveolar and interstitial fluid) of the lung, metallic nickel alloys have very limited potential to release nickel ions that might have the capacity to damage cells or interact with DNA to cause cancer.

For example, findings of two-year cancer inhalation bioassays in rats support this theory, as they demonstrate no carcinogenic potential for metallic nickel or water-soluble nickel sulfate; water-insoluble nickel subsulfide showed a clear carcinogenic effect, while water-insoluble nickel oxide presented equivocal results (Oller et al., 2008; NTP 1996a-c). Figure 1, from Goodman et al. (2011), provides a graphical representation of the nickel ion bioavailability model. These findings support that nickel substances, including metallic nickel, cannot simply be assessed based on water solubility. Conversely, bioaccessibility in synthetic lung fluids can provide key information for determining the potential for toxicity and/or carcinogenicity relative to other nickel-containing materials. While information on factors such as the rate of cellular uptake remain unknown, estimating the bioaccessible fraction in interstitial/alveolar and lysosomal fluids can provide important perspective regarding the potential extra- and intracellular release, respectively, and ultimately can inform the potential for adverse respiratory effects, as described in Figure 1. With regard to Figure 1, note that nickel subsulfide, nickel oxide, and metallic nickel are all water insoluble, while nickel sulfate heptahydrate is freely water soluble.

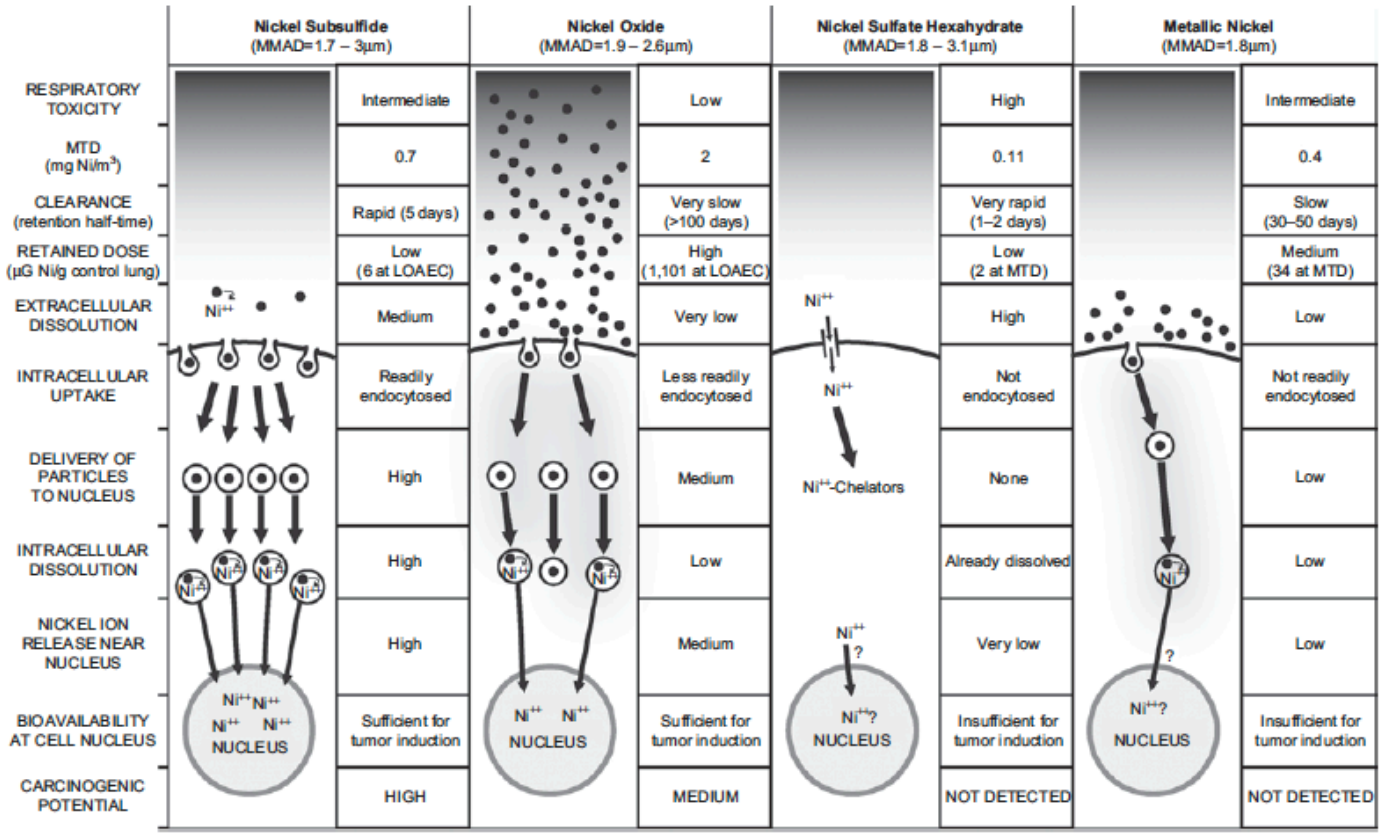

Figure 1. The nickel ion bioavailability model takes into account the various factors that determine the bioavailability of the nickel ion at the nucleus of target cells *in vivo*. The examples in this figure are based on the results of *in vitro* and *in vivo* mechanistic studies as well as the rat inhalation bioassays with nickel-containing substances.

Figure 1. Nickel ion bioavailability model (figure from Goodman et al. 2011)

As shown in Figure 1, for nickel to pose a cancer hazard, nickel ions need to be released in proximity to the nucleus. Nickel in alloys should be evaluated separately from nickel compounds (e.g., nickel subsulfide, nickel oxide) and nickel metal, because although insoluble in water, they do not dissolve to release nickel metal ions on exposure in the same manner as nickel subsulfide and nickel oxide (quantitative discussion of these findings follows).

3. Toxicity and Bioavailability of Nickel from Alloys

Alloys are unique materials, defined as metals or metalloids combined with other intentionally added elements in such a way that the elements cannot be separated. The United Nations (UN 2013) defines an alloy as "a metallic material, homogeneous on a macroscopic scale, consisting of two or more elements so combined that they cannot be readily separated by mechanical means." Alloys are designed to attain specific technical, mechanical, physical, or chemical properties that cannot be accomplished by the

individual alloy constituents. These effects are due to changes in the microstructure of the materials, resulting in bonds that change the physical-chemical nature of the metal constituents in a way that differs from their pure metallic state. For example, the amount of metal ion release from a metal such as nickel in an alloy can be significantly different from the amount released from the stand-alone metal (e.g., metallic nickel). Elemental metals in the matrix of a chromium-rich alloy (e.g., stainless steel) are known to have dramatically different potential bioavailability (defined as the fraction of the dose that reaches systemic circulation) and toxicity.

The reason for such differences in bioavailability is the amount and extent of metal ion released from the alloys in bodily fluids, and thus made available for absorption and interaction with target tissues/sites. It is the metal ion at the target site that has been demonstrated to be the main factor in determining the toxic potential of metals. The inhalation toxicity of nickel metal following repeated exposure has been evaluated and reported (Oller et al., 2008; discussed above). However, the same potential for toxicity of nickel metal in an alloy cannot be assumed, because the bioavailability of nickel metal from the alloy differs. In most cases, the ability of metal ions to dissociate and be released from alloys in physiological media (e.g., lung fluid) has been shown to be decreased in alloys (examples discussed below). Factors that affect the release of nickel metal, and subsequently the bioavailability, include surface oxide characteristics, physical form, particle size, surface area, thermal history, physiological conditions, and matrix effects (Eurometaux, 2015; Oller et al., 2015; Stockmann-Juvuala et al., 2013). As a result, the potential for toxicity of an alloy cannot be predicted simply by the concentrations of its metal constituents.

For example, Inconel 716, 7-14, and WASP are metal alloys containing chromium, cobalt, and nickel, which are at least 15% elemental chromium. It is known that alloys such as these have, a trivalent chromium surface layer that is reasonably impervious to chemical reactions, and this characteristic is maintained even when the alloy is ground or abraded. This chromium oxide layer does not allow for significant release of metal ions in biological fluids (Herting et al., 2008; Stockmann-Juvala et al. 2013; Hillwalker and Anderson, 2014). This is why stainless steel, and other such alloys containing high levels of chromium, resist corrosion, and why they are non-reactive or soluble in biological media.

In fact, the lower inhalation toxicity of a nickel-containing stainless-steel alloy (SS316L; 10% Ni, 18% Cr, 2% Mo, 70% Fe) relative to its nickel metal content was demonstrated in a 28-day study in rats (Stockmann-Juvuala et al., 2013). In this study, the SS316 powder was shown to have a no-observed-adverse-effect concentration (NOAEC) ≥1,000 mg SS316/m^3. This is in contrast to a lowest-observed–adverse-effect concentration (LOAEC) of 0.4 mg nickel/m^3 identified in a 28-day inhalation toxicity range-finding study of nickel metal powder (Oller et al., 2008, 2015). Simply based on the nickel content of the alloy (10%), one might predict its toxicity to be only 10-fold lower than that of a pure nickel metal; however, these studies demonstrate that the properties of the nickel metal, and its relative content, do not directly correlate to the properties of the alloy. Rather, the reduced toxicity is a reflection of the reduced bioavailability of the

nickel ion from the alloy. This concept is further supported by bioaccessibility data reported in the same study, discussed below (Stockmann-Juvuala et al., 2013).

4. Bioaccessibility

Bioaccessibility is measured as the *in vitro* dissolution in synthetic biological fluids, which is a surrogate for the amount of a substance (e.g., metal ion) available for absorption, e.g. for bioavailability (EPA, 2007c; Henderson et al., 2012; Ruby et al., 1999). As discussed above, this fraction, or bioaccessible concentration, has been shown to be a more accurate predictor of potential for toxicity to human health than basing assessments on metal concentration alone (Eurometaux, 2015: Henderson et al., 2012, 2014; Oller et al., 2015).

The degree of bioaccessibility depends on the chemical form (i.e., species) and presence of other elements. Because toxicity criteria typically are developed for metals in a freely soluble and bioaccessible form, exposure estimates for metals are frequently refined for site-specific conditions by accounting for bioaccessibility. *In vitro* tests measure the amount of metals released from a given material into fluids designed to mimic those of the human body (e.g., synthetic gastric fluid to simulate oral exposure, or synthetic lung fluids to simulate inhalation exposure) (Ruby et al., 1999; Henderson et al., 2012). The metals dissolved in these fluids may become available for uptake, and as such, these assays provide a conservative estimate of bioavailability.

The bioaccessibility/bioavailability concept was demonstrated in the Stockmann-Juvuala et al. (2013) study discussed above, which was designed to determine whether bioaccessibility data could predict the toxicity of alloys, using stainless steel as an example. In this study, nickel ion release from the SS316 alloy in simulated lysosomal fluid was shown to be 1,000-fold less than release from a nickel metal powder in the same assay, suggesting that inhalation toxicity would be 100-fold lower based on the relative bioaccessible fraction (Eurometaux, 2015; Oller et al., 2015; Stockmann-Juvuala et al., 2013). As discussed above, this finding was confirmed *in vivo* where the inhalation toxicity in rats was at least 25-fold lower than would have been if predicted based on the nickel content alone.

While water solubility is often used to differentiate and predict toxicity between nickel compounds, this approach of using bioaccessibility in relevant biological fluids has been shown to be a more robust method for assessing the potential bioavailable fraction and related toxicity of metals, including nickel (Henderson et al., 2012; OECD; 2014). The document provided by Eurometaux (2015) provides an exhaustive summary of published studies using bioaccessibility to assess metals, including those that correlate findings with *in vivo* data. Of note, the metals industry is in the process of seeking validation at the OECD level for the bioaccessibility methods used in many of these studies; the OECD standard project submission form (SPSF) has been completed, and discussions are ongoing with possible lead countries.

The EPA has also issued guidance for evaluating bioaccessibility and bioavailability of metals in soil and encourages the incorporation of these concepts in risk assessment (EPA 2007a,b,c, 2009). For example, EPA's guidance on metals risk assessment offers the following conclusions:

> "The form of the metal (chemical species, compound, matrix, and particle size) influences the metal's bioaccessibility, bioavailability, fate, and effects" EPA (2007c).
>
> "As feasible, inhalation exposure estimates should be specific to the metal speciation in order that there can be pertinent correspondence with the form of the metal used for the dose-response assessment (e.g., in deriving the RfC or IUR estimate)" EPA (2007c).

5. Bioaccessibility of Metal Ions from Alloys

Bioaccessibility measures for metals in chromium-rich alloys have been published in the scientific literature. It has been shown that metals present in alloys have very limited bioaccessibility and that the metal release from alloys cannot usually be predicted based solely on composition. As discussed above, it has been shown that the inhalation toxicity of nickel in some stainless steels cannot be estimated accurately on the basis of toxicity data for metal compounds, or even for metals in the pure elemental form (Stockmann-Juvala et al. 2013).

In another study, the bioaccessible fraction of chromium, manganese, and nickel from Inconel (a nickel alloy), in artificial lysosomal fluid (ALF), representative of the target tissue of the lung, was reported to be only 0.149%, 0.73%, and 0.10%, respectively (Hillwalker and Anderson, 2014). Similarly, bioaccessibility of cobalt from stainless steel 304 was not measurable (<0.00027%) and for chromium, manganese, and nickel from stainless steel 304 particles in ALF was also extremely low (only 0.13%, 0.44%, and 1.8%, respectively) (Hillwalker and Anderson, 2014). By comparison, the bioaccessibility of pure elemental cobalt, manganese, and nickel in ALF has been shown to be 30%, 20%, and 25%, respectively (Hillwalker and Anderson, 2014). In a separate study, the bioaccessible concentration of nickel from an Inconel alloy in lysosomal fluid was found to range from 0.05% to 0.4% (Henderson et al., 2014). Because elemental metals in alloys are not readily solubilized in ALF, they do not pose a significant human health hazard via inhalation, as exemplified by work specific to nickel metals and nickel-containing stainless steels (Stockmann-Juvala et al. 2013; Goodman et al. 2011). As shown by Stockmann-Juvala (2013) and colleagues, available data demonstrate the lack of respiratory toxicity in an animal model of a nickel-containing stainless-steel alloy, as would be expected based on bioaccessibility analysis.

This concept has also been shown to be true for metals in simulated gastric fluid (EPA 2007a,b; 2009; 2012; Henderson et al., 2012). Although most of EPA's work on bioaccessibility has been specific to lead and arsenic in soil and mine tailings, the concept is also applicable to other metals and other media. The bioaccessibility of chromium,

manganese, and nickel, bound in Inconel, in simulated gastric fluid, is only 0.0523%, not measurable (<0.00007%), and 0.067%, respectively (Hillwalker and Anderson, 2014). Similarly, that of cobalt in stainless steel 304 is not measurable (<0.00027%), and the bioaccessibility of chromium, manganese, and nickel from stainless steel 304 particles in simulated gastric fluid is also extremely low, only 0.018%, <0.00007%, and 0.10%, respectively (Hillwalker and Anderson, 2014). By comparison, the bioaccessibility of pure elemental cobalt, manganese, and nickel in gastric fluid is 16%, 73%, and 0.95%, respectively (Hillwalker and Anderson, 2014).

Other regulatory agencies and standards worldwide have also started to incorporate this concept for alloys, including those containing nickel (European Commission, 2008, 2013; BS EN 1811, 2011; BS EN 71-3, 2013; European Council, 2006). For example, while nickel metal is classified as a dermal sensitizer in the European Union, the Classification, Labeling and Packaging of Substances and Mixtures (CLP) Regulation allows for classification of nickel-containing alloys based on the release of nickel metal ion, as opposed to their nickel content (European Commission, 2008). In addition, many industry and research groups have begun applying this concept to hazard and risk identification of alloys; this approach uses information on metal ion release to refine assessments (Eurometaux, 2015: Henderson et al., 2014; Oller et al., 2015). The global metal industry has been working over the last several years to develop a guidance document for performing assessments on alloys as part of their Health Risk Assessment Guidance for Metals (HERAG) series (https://www.icmm.com/page/1213/health-risk-assessment-guidance-for-metals-herag). This document, titled, *HERAG ALLOYS FACT SHEET: Hazard identification and classification of alloys for human health endpoints*, is still in working-draft form. However, an excerpt has been provided to the European Commission and EU Member States for discussion and is provided in Attachment 1 because it is not publically available. This document provides approaches for toxicity classification for alloys developed by the metals industry using information on the characterization of the alloy, bioelution or bioaccessibility testing and read-across approaches (Eurometaux, 2015). Although these approaches are not finalized and do not specifically provide toxicity criteria for alloys, it is insightful as to approaches to assess the potential hazard posed by metals in alloys.

It is also noteworthy that, while "nickel and nickel compounds" are listed as inhalation carcinogens under California's Proposition 65, OEHHA (2004) specifically exempted nickel in alloy form from the regulation, stating:

> "For the purposes of clarification, OEHHA notes that nickel alloys are distinct from nickel compounds, and are not included in the Proposition 65 listing of *nickel compounds*. A nickel compound is a substance consisting of nickel and one or more other elements combined in definite proportions (e.g., by ionic or covalent bonds). A nickel alloy is a mixture of nickel with one or more other elements, typically produced by mixing molten nickel with other substances. The atoms in an alloy are not covalently or ionically bonded in fixed ratios."

Because the potential health effects associated with exposure to elemental metals, especially those bound in an alloy, can differ substantially from those of metal

compounds, the practice of treating elemental metal as metal compounds distorts the characterization of metal toxicity. Furthermore, when the elemental forms exist as an alloy, the potential for toxicity is often highly diminished, to the point of being negligible. By applying toxicity weights for bioavailable metal compounds to the metal content of alloys, the scores for elemental metals as alloys are substantially exaggerated. Rather, the bioavailable fraction of the metal constituents, such as nickel, should be ascertained and used to refine any assessment. To do this, bioaccessibility methods have been used increasingly to describe the relative release of metal ions from alloys, in order to allow for more refined hazard and risk assessments.

6. Epidemiologic Data Review for Occupational Exposure to Nickel Alloys

Studies of nickel refinery workers have demonstrated that a significant increased risk of lung cancer[2] exists in this industry historically (IARC, 2012; ATSDR, 2005). Exposures in this industry include nickel subsulfide, nickel oxide, and nickel refinery dust, which is a mixture of many nickel moieties, including nickel sulfate, and the exact carcinogenic nickel forms in nickel refinery dust are not specifically known (EPA, 1987). The inhalation cancer unit risk factor for nickel refinery dust is used to set the ABC value for insoluble nickel. As discussed above, nickel alloys, though insoluble in water, should not be evaluated using the nickel refinery dust unit risk factor or insoluble ABC value, because the forms of nickel in nickel refinery dust are chemically and toxicologically different from the nickel in alloys, due to limited bioavailability and bioaccessibility in the lung.

Several studies of nickel alloy workers have also been conducted internationally, and it is appropriate to consider whether the findings of these studies support the observation from other lines of evidence that nickel in alloys should not pose a carcinogenic hazard in humans. Overall, consistent with the findings of other reviews (IARC, 2012; ATSDR 2005; NTP 2013), we find that the epidemiologic data for nickel alloy workers do not support an increased risk of lung cancer associated with occupational exposure to nickel in the alloy form.

ATSDR stated:

> "In contrast to the findings of nickel refinery workers, most studies in other groups of nickel workers have **not found significant increases in the risk of lung cancer** among: workers employed in nickel mining and smelting facilities (International Committee on Nickel Carcinogenesis in Man 1990; Shannon et al. 1984b, 1991), workers employed at a hydrometallurgical refinery (Egedahl and Rice 1984, Egedahl et al. 1991, 2001), **workers employed at nickel alloy and**

[2] Other than lung and nasal cancers, the epidemiologic evidence that nickel exposure can cause cancer at other sites is inconsistent and limited (IARC, 2012). As such, the assessment herein is focused on respiratory cancers—specifically, lung and nasal cancers.

stainless steel production facilities (Cornell 1984; Cornell and Landis 1984; Cox et al. 1981; Enterline and March 1982; International Committee on Nickel Carcinogenesis in Man 1990; Jakobsson et al. 1997; Moulin et al. 1993; Sorahan 2004), workers employed as stainless steel welders (Danielsen et al. 1996; Gerin et al. 1993; Hansen et al. 1996; Simonato et al. 1991), workers involved in nickel-chromium electroplating (Pang et al. 1996), or workers employed at a barrier production facility (Cragle et al. 1984; Godbold and Tompkins 1979; International Committee on Nickel Carcinogenesis in Man 1990). Although some studies of these workers did find significant increases in respiratory tract cancers (Becker 1999; Moulin et al. 1990), the increased risk was attributed to exposure to other carcinogenic agents, such as polycyclic aromatic hydrocarbons or asbestos (p 80)." [emphasis added]

In synthesizing the epidemiologic data, IARC concluded that "there is an elevated risk of lung and nasal sinus cancer among nickel refinery workers (IARC, 1990; Anderson et al. 1996; Anttila et al. 1998; Grimsrud and Peto, 2006), and an elevation in lung cancer risk among nickel smelter workers (IARC, 1990; Anttila et al. 1998)" (p. 190, IARC, 2012). Similar to ATSDR and NTP, IARC did not identify nickel alloy and stainless steel workers having elevated respiratory cancer risk from nickel exposure.

Table 1 summarizes the results from studies of workers exposed to nickel-containing alloys, noting also limitations of each study. Studies of stainless-steel welders were excluded from the assessment, because they were also exposed to hexavalent chromium and other compounds, and it is not possible to differentiate the potential risk posed by nickel (IARC, 2012). Only one study reported an increased risk of lung cancer among alloy workers (Arena et al., 1998). This study evaluated more than 31,000 workers in thirteen US high-nickel alloy plants. Although an increased lung cancer risk was found when compared to the general US population, the risk was not significant when the expected rate was calculated from the local metropolitan areas where the plants were located (Arena et al., 1998) (Table 1). Regarding the findings of Arena et al. (1998), IARC noted that the lung cancer standardized mortality ratio (SMR) was elevated and provide some association between exposure in these plants and lung cancer. However, IARC also added that "primary exposure was to nickel oxide and thus, the study cannot be used to evaluate the specific carcinogenicity of metallic nickel. Analysis of lung cancer by duration of employment did not indicate a dose-response" (p. 187, IARC, 2012).

The insoluble nickel ABC value, which is based on exposure to nickel refinery dust, is not appropriate to assess the potential hazards and cancer risk of nickel in alloy form. ATSDR described that nickel refinery workers were exposed to high levels of sulfidic and oxidic nickel and low levels of soluble and metallic nickel in comparison to nickel workers of other industries (ATSDR, 2005). Although it can be difficult to assess the potential hazards posed by nickel alloy alone using epidemiology data, because the exposures are mixed, the epidemiological evidence does not support a significant risk associated with worker exposure to nickel in the form of alloys, even at very high concentrations (e.g., mg/m^3, Table 1), relative to environmental exposures to nickel, which are typically in the range of ng/m^3.

7. Conclusions on the Potential Cancer Hazard Posed by Nickel Alloys

In conclusion, multiple lines of evidence described herein support a conclusion that nickel in the form of alloys does not pose a cancer hazard that can be evaluated or quantified based on data for insoluble nickel exposures in the refining industry or nickel refinery dust specifically. As such, the ODEQ insoluble nickel ABC should not be applied to assess the potential hazard posed by nickel in an alloy. Nickel in alloys should be assessed separately from insoluble nickel compounds, because as not doing so substantially exaggerates the potential risk. This finding is consistent with those of internationally recognized agencies and governmental regulatory authorities (NTP, 2013; ATSDR, 2005; IARC, 2012; OEHHA, 2004).

Table 1. Epidemiologic studies of stainless-steel and nickel-alloy workers

Author/ Year	Study Population	Inclusion Criteria	Study Type/Methods	Nickel Exposure Reconstruction	Risk Estimates	Limitations and Other Findings
Arena et al. (1998)	31,165 workers from 13 high-nickel-alloy plants located throughout the US	Each case employee had worked a minimum of 1 year within the interval 1956 through 1967	Cohort mortality study — Compared mortality of workers to the total US population and local population in proximity to the plants	Measures of cumulative time employed in the high-nickel-alloy industry or specific work area were used as a crude surrogate of cumulative exposure Average concentration range: 0.0064 to 1.5 mg/m^3	Lung cancer SMR for white males (observed deaths = 831) Compared to US population—SMR 1.13 (95% CI 1.05 to 1.21) Significantly increased Compared to local populations—SMR 1.02 (95% CI 0.96 to 1.10)—consistent with expected Two nasal sinus cancers were reported[a]	Risk estimates indicating an elevated lung cancer risk depended on the population used as the comparator. Analysis of lung cancer risk by duration of employment and time since first employment did not show exposure-response relationships IARC noted that exposures included oxidic nickel
Cornell (1983)	Former workers at plants of seven companies in the US engaged in the production of stainless and low-nickel-alloy steels (n=4882 deaths)	All deaths were included for at least the 5-year period up to the end of 1977	Proportional mortality study — Comparisons of proportional mortality rates instead of death rates were carried out because of lack of information	Exposure reconstruction was not performed. 3,343 (74%) of deaths were among men who were potentially exposed to nickel during their work	SPMR = 0.97—Consistent with expected (218 observed deaths for malignant neoplasms of bronchus, lung, trachea, among those potentially exposed to nickel) No nasal cancers were observed	Proportional mortality study does not measure the risk of deaths from cause. It measures only the relative frequency of the particular cause of deaths among all causes of death No data on nickel exposure

Table 1. (continued)

Author/ Year	Study Population	Inclusion Criteria	Study Type/Methods	Nickel Exposure Reconstruction	Risk Estimates	Limitations and Other Findings
Cox et al. (1981)	1,925 workers in a plant in Britain manufacturing nickel alloys from raw materials consisting of metallic nickel and other metals	Males employed in the operating areas of the plant for a total of 5 years or more before April 1, 1978 Males who were untraced at April 1, 1978, or who emigrated were excluded	Cohort mortality study—Compared mortality of workers to the number of expected deaths for the male residents of Herefordshire towns aged 15 to 64 years, 1969 to 1973	Measurements of the facility have been done systematically since 1975. Samples have been taken by Casella samplers pinned to the coat lapels of workers working over periods of 2, 4, or 8 hours Average nickel concentrations in 1975 to 1980: 0.04 to 0.84 mg/m^3	Lung cancer SMR = 87 (observed deaths = 15) — Consistent with expected No death due to nasal cancer	According to the authors, only 117 deaths in total were recorded, with 15 being from lung cancer. Overall mortality was significantly low and may be attributed partly to the selection of healthy men Risk of death from respiratory disease other than cancer was well below the normal risk for the locality. It is possible the men susceptible to chronic respiratory disease may have been excluded by the pre-employment medical examination or by self-selection It was not possible to obtain smoking data for the workers

Table 1. (continued)

Author/ Year	Study Population	Inclusion Criteria	Study Type/Methods	Nickel Exposure Reconstruction	Risk Estimates	Limitations and Other Findings
Jakobsson et al. (1997)	823 workers exposed to the dust of grinding materials, grinding agents, and stainless steel at two plants in Sweden	All men employed at least 12 months during the period 1927 through March 1981 were included	Retrospective cohort study SMRs and SIRs were calculated for cause-specific mortality between 1952 and 1993, and for cancer morbidity between 1958 and 1992	Authors described that workers were exposed to metal dust (stainless steel; 18% nickel, 8% chromium) and dust from abrasives Measurements in the second plant for 1975 to 1980 indicated dust levels at grinding of 0.7 to 7.3 mg/m^3 (3%–10% chromium, 2%–5% nickel) and at brushing and polishing 1.6 to 16 mg/m^3 (1% chromium, 0.5% nickel)	Lung cancer SIR = 0.6 (95% CI 0.2 to 1.2) based on seven observed cases-Consistent with expected No death due to sinonasal cancer	According to the authors, healthy worker selection is possible with reduced overall mortality including cardiovascular and tumor mortality Information on smoking was not available (however, low risk of lung, larynx, uroepithelial, and pancreatic cancers indicate low consumption of tobacco)
Moulin et al. (1993)	4,227 workers in a stainless-steel plant in France	All male workers who were employed at the plant January 1968 through December 1984. Cohort was restricted to workers having at least 3 years of employment	Cohort study—Mortality patterns were studied from 1968 to 1984. Observed numbers of deaths were assumed to follow Poisson distribution, and 95% CIs were computed for SMRs	Job titles describing all jobs performed by each worker were listed and coded, along with dates of beginning and end of each occupation No measurements were available on the industrial process, and thus, no quantitative exposure assessment was conducted	Lung cancer SMR = 1.32 (95% CI 0.94 to 1.80) based on 39 deaths—Consistent with expected Lung cancer SMRs were reported according to specific workshops: *all workshops non-significant except in the foundry — SMR = 2.29 (95% CI 1.14 to 4.09) based on 11 deaths—Significantly increased	Healthy worker effect was possibly present. Author indicated that the health hazards in stainless-steel foundry operations may include exposure to silica, metal fumes (Fe, Cr, Ni), and degradation products from molds and cores (PAH, carbon monoxide, formaldehyde)

Table 1. (continued)

Author/ Year	Study Population	Inclusion Criteria	Study Type/Methods	Nickel Exposure Reconstruction	Risk Estimates	Limitations and Other Findings
Moulin et al. (2000)	4,897 workers in France involved in the production of stainless and alloyed steel from 1968 to 1992	All workers ever employed at the plant for at least 1 year during January 1968 through December 1991	Cohort mortality study and nested case-control study Follow-up period for mortality lasted from January 1968, or the date of first employment if later, through December 1992	No airborne exposure-level measurements were available All exposure-parameter estimates were based on the experts' subjective quantification	Lung cancer SMR = 1.19 (95% CI 0.14 to 4.61) based on 54 observed deaths—Consistent with expected Crude and smoking-adjusted ORs of lung cancer associated with employment in workshops were all non-significant except for ring manufacture (6 cases and 3 controls): Crude and adjusted ORs with workers exposed to Cr and/or nickel were non-significant	Cross-sectional definition of the study population (workers active at January 1, 1968) may have introduced a survivor selection Nested case-control design likely accounted for the healthy survivor effect Exposure assessment was based on a job exposure matrix. Exposure measurements were not available Due to simultaneous exposure, it was difficult to distinguish the independent effect of each chemical

Table 1. (continued)

Author/ Year	Study Population	Inclusion Criteria	Study Type/Methods	Nickel Exposure Reconstruction	Risk Estimates	Limitations and Other Findings
Sorahan et al. (2004)	1999 workers at the plant manufacturing nickel alloys in Hereford, Britain	All male workers who were first employed in the factory environment of the Hereford nickel alloy works in the period 1953–1992 (excluded office staff, canteen staff, laboratory workers and medical staff), and who were employed in the factory environment of the alloy works for a minimum period of 5 years	Updated cohort mortality study Observed mortality rates compared to male mortality rates for England and Wales Poisson regression was used to calculate risk ratios for lung cancer	Workers were not exposed to nickel subsulfide, but would have had some exposure to iron, copper, cobalt, trivalent chromium compounds, and molybdenum Measurements of total inhalable dust (all in mg/m^3) — Unadjusted means, 1975 to 1980, for nickel ranged from 0.04 to 0.84 Unadjusted means, 1997 to 2001, for nickel ranged from 0.29 to 0.45 8-h TWA, 1997 to 2001, ranged from 0.16 to 0.33	SMR for lung cancer = 0.87 (95% CI 0.67 to 1.11) based on 64 observed deaths — Consistent with expected Lung cancer mortality by period from first employment, 1958 to 2000 — all were non-significant results Lung cancer mortality by operator areas of first job — all were non-significant results Lung cancer risk in relation to duration of employment — all were non-significant results No death from nasal cancer in the cohort	Authors indicated that it was not possible to compile a reliable job exposure matrix for all periods of interest, and thus, analyses in relation to cumulative Ni exposure were not attempted There was evidence of healthy worker effect

Table 1. (continued)

Author/ Year	Study Population	Inclusion Criteria	Study Type/Methods	Nickel Exposure Reconstruction	Risk Estimates	Limitations and Other Findings
Svenssen et al. (1989)	1,164 males in Sweden who handled stainless steel in two manufacturing plants (grinding, finishing, polishing occurred)	Workers were employed for at least 3 months during the period 1927–1981	Cohort mortality study Vital status determined up to December 31, 1983 Observed deaths compared to the mortality rates for males in Blekinge County, Sweden	1st plant — no measurements were made 2nd plant — Several measurements in the period 1975–1980 showed dust levels at grinding of 0.7 to 7.3 mg/m^3 (3%–10% chromium, 2.5% nickel) and at brushing and polishing of 1.6 to 15.7 mg/m^3 (1% chromium, 0.5% nickel) Grinders and brushers/polishers were considered to have high exposures to total dust, chromium, and nickel	Lung cancer SMR =0.92 (95% CI 0.44 to 1.79) based on 9 deaths—Consistent with expected For those with 5+ years of exposure and 20+ years of latency period: Lung cancer SMR = 0.55 (95% CI 0.09 to 2.14) based on two observed deaths One death due to nasal cancer	Healthy worker effect was noted

OR: odds ratio

SIR: standard incidence ratio

SMR: standardized mortality ratio

SPMR: Age-standardized proportional mortality ratio

8-h TWA: 8-hour time-weighted average

[a] The authors combined the two nasal sinus cancer deaths with one mediastinum cancer death and analyzed as "other respiratory cancer." Risk was reported only for white males, and it was not elevated when compared to the US and local populations; RR = 0.35 (95% CI 0.05 to 1.02); RR = 0.32 (95% CI 0.10 to 1.00).

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APPENDIX 1

Excerpt from HERAG Alloys Fact Sheet

Eurometaux
European Association of Metals

DOC/03

Preparatory material for the 5 October meeting on
"Biological availability in the framework of art. 12 (b) of the Regulation N° 1272/2008
on Classification Labelling and Packaging of substances and mixtures"

This package aims to address the issues/concerns identified by the participants at the 27 April meeting (see also pages 8-9 of Doc. CA/58/2015, circulated on 15 June 2015)

It includes:

Documents	**What?**	**Pages…**
Industry note	This note was revised in line with the comments made at the 27 April meeting	3
Annexes		
One-pager 1	Comparison of default and proposed approach including conservatism	39
One-pager 2	Reference material: choice/justification/information	43
One-pager 3	Representativity of the fluids	45
One-pager 4	Correlation in vitro-in vivo	49
One-pager 5	Sequential vs. parallel testing	57
One-pager 6	Enforceability	61
SPSF	Completed SPSF template	Annex SPSF
SOP	Revised SOP	Annex SOP

Industry note on classification of alloys for human health endpoints /use of bioelution data/protocols

Update September 15, 2015

This note, prepared in view of the expert meeting organised by the Commission on 27 April 2015, has been updated and revised in view of the follow-up meeting of October 5 2015.It compiles extracts from two industry documents:

- The **HERAG (Health Risk Assessment Guidance) Alloys Fact sheet** addressing "hazard identification and classification of alloys for human health endpoints". This fact sheet has been prepared in view of the CLP/GHS mixtures deadlines.

- The **"bioelution roadmap (applications and use of "bioelution" approaches for metals, inorganic metal compounds and complex materials containing metal)"** prepared by industry in 2014 in view of the Stakeholders workshop organised on May 22 2014. The document has been revised to consider the comments expressed by the participants. It aims at explaining and clarifying what is meant by 'bioelution', what its potential applications are, how to use bioelution results in practice and what the required work is to back up/validate its uses.

It is accompanied by a series of 'one-pagers' addressing more in detail technical issues and concerns raised at the 27 April meeting.

Two additional documents are included in the package:

- The Standard Operating Procedure (SOP) for the actual bioelution testing as reported in the publication of Henderson et al. 2014 has now been improved/clarified to take into consideration several protocol-related aspects raised during the discussions with authorities in 2014. The scope of the current SOP is specifically developed for bioelution testing in fluids designed to determine alloys bioaccessibility related to oral exposure and systemic effects.

- The OECD standard project submission form (SPSF) has been completed, as an attempt to facilitate discussions with possible lead countries (National Coordinators)

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1. INTRODUCTION

1.1 Background and scope

Alloys are a prominent and diverse group of materials. While some metals are used as engineering materials or in consumer products in their elemental form or as simple mineral compounds, the majority of metals in commerce are used in the form of alloys. An alloy can be defined as "…*a metallic material, homogeneous on a macroscopic scale, consisting of two or more elements so combined that they cannot be readily separated by mechanical means*" (UN, 2013).
Alloys appear on the market in large volumes and mainly in massive form and powder forms; ultrafine or nano forms also exist. Just as with pure substances, appropriate chemical management practices for such materials are essential and the mining and metals industry is committed to developing and implementing good practice in this regard.

Several regulatory frameworks require robust assessment of mixtures for example, the UN Globally Harmonised System of Classification and Labelling (GHS) (UN 2003, 2005, 2007, 2009, 2011, 2013) and in the European Union (EU) Registration Evaluation and Authorisation of Chemicals Regulation (REACH, Regulation EC No 1907/2006). These frameworks require hazard identification, classification and exposure scenarios to be developed and circulated throughout the supply/use chain for mixtures, including their use in downstream applications. The implementation deadline of the European Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP) for mixtures is 1 June 2015. This means that classifications for all the mixtures in commerce must be compliant with CLP terminology and communicated to the supply chain (via Safety Data Sheets) as of 1 June 2015.

In general, a metallic alloy consists of a metal or a metalloid base element, constituting the largest percentage of the material and one or more intentionally added elements to achieve specific and improved mechanical, physical or chemical properties compared to the alloy's individual constituents. Alloys are typically designed to have different properties than those of their constituent metals (e.g., to make them less reactive, more durable, provide greater strength or improve machinability).

In the follow-up of the expert meeting of 27 April, the scope of this note has been revised and focuses on the use of bioelution protocols/data for classification of systemic effects after oral exposure.

1.2 Summary of alloy properties

Mechanical or chemical properties of pure metals are in many cases not sufficient for a specific application as the metal may be too soft, too brittle or have insufficient corrosion resistance. A modification of intrinsic properties can be obtained through alloying. This includes changes in microstructure and improved mechanical properties, biocompatibility and corrosion resistance compared to the constituent materials.

Alloying elements can be present in an alloy in different ways depending on the material and the microstructure. Most alloys are so called 'solid solution alloys' where the alloying constituent(s) are dissolved into the crystal structure of the base metal, substitutionally or interstitially. In substitutional solid solutions, some of the atoms of the base metal are substituted for atoms of the alloying constituent. In interstitial solid solutions, atoms of the alloying element are placed in the small sites between the atoms of the base metal. Interstitial and substitutional atoms can be present simultaneously in the same alloy. In some alloys, metallic alloying elements are dissolved as long as the alloy is in its liquid form but precipitates during cooling. This type of alloy is denominated as an immiscible alloy.

An important property of most alloys is their insolubility in aqueous solutions (Dresher and Poirier, 1997). Alloys, however, react by **corrosion** in air and aqueous media to form new metal-containing species that may or may not be readily soluble. Corrosion is a natural process by which the metal or alloy thermodynamically strives to reach its most stable state for a given environment. It is a complex process where several factors modulate the extent of metal release from the material. These include the instantaneous formation of surface oxides with passive properties or the formation of a surface layer of oxidised material that acts as a barrier and hinders further corrosion to a different extent. Their properties influence the availability of constituent metals in alloys for exposure and potential toxicological effects in humans or the environment.

In contrast to corrosion, which is primarily an electrochemical process, the process of **metal release/dissolution** usually involves multiple-step chemical and electrochemical reactions. The main interface for the metal release process during a specific exposure condition is between the oxide, or layer of corrosion products, on the metal/alloy surface and the surrounding environment. Thus, metal dissolution can be described as the amount of metal that is released or dissolved from surface oxides or corrosion products on metals or alloys per units of surface area and time (Leygraf and Graedel, 2002). As corrosion and metal release are two processes governed by different mechanisms, corrosion resistance and corrosion rate data cannot be used in isolation to predict the extent of metal release.

The extent of metal release from metallic alloys can be significantly different in comparison with their pure metal constituents. It is therefore imperative to examine if alloys behave as unique materials of disparate intrinsic properties or as simple mixtures of their pure constituents (Herting et al., 2005, 2008a; Goidanich et al., 2008; Stockmann-Juvala et al., 2013; Hornez et al., 2002).

The assumption is that all substances in alloys are considered to be **bioavailable** to some extent. However, the release rate, uptake, and toxicity of constituent metals (i.e., bioavailability of the metal constituents from which alloys are composed) are related to the surface oxide characteristics, which govern the extent of released metals ions in contact with water/biological fluids, soil and air. Other

factors that will affect the bioavailability of the alloy constituents include the physical form and the inclusion into a matrix or complex structure. These aspects can also be described by the terms metal **bioaccessibility** and **bioelution**, which are defined in Box 1 below.

Box 1: Definition of Bioavailability, Bioaccessibility and Bioelution

Bioavailability is defined as the extent to which a substance is taken up by an organism and is available for metabolism and interaction. For local effects, bioavailability refers to the extent to which the chemical can reach its target site at first point of contact/entry. The systemic toxicity of most metals and metalloids is associated to a large degree with the release of soluble metal ions and their uptake by the body and/or interaction at their target organ sites (i.e., the bioavailability of the metal ions). Therefore, the bioavailability of most metals is defined as the extent to which the soluble metal ion can be available at the target organ/site.

Bioaccessibility is defined as the fraction of a substance that dissolves under surrogate physiological conditions and therefore is potentially available for absorption into systemic circulation (systemic effects) or for interaction at port of entry sites (local effects). Bioavailability will depend on release of the metal ion (as a necessary first step) and further uptake of the soluble fraction; thus bioaccessibility can be considered as a conservative estimate of bioavailability for metals.

Bioelution refers to the *in vitro* methods used to measure the degree to which a substance (e.g., metal ion) is released in artificial biological fluids. Such tests are thus used to estimate a substance's bioaccessibility in the form of released metal ions (i.e., its solubility under physiological conditions).

These concepts are discussed in more detail in Annex 1.

The generation of accurate bioaccessibility data requires reproducible in-vitro tests on alloys as placed on the market (this includes their physical form, shape, size and surface characteristics) and relevant exposure conditions. Bioelution testing is conducted on materials (e.g., metals, alloys, etc.) by using different protocols and approaches to measure the extent of metal release/dissolution in synthetic biological fluids, thereby determining the fraction of bioaccessible metals. Examples of such protocols are reported in various publications (e.g., EN71-3, 1995 and EN71-3, 2013, CEN, 1998; ASTM, 2003; Stopford et al., 2003; Herting et al., 2008a; Midander et al., 2010; Hedberg et al., 2013; Henderson et al., 2014). The chemical composition of the synthetic biological fluid and exposure conditions of the bioelution method used are to be good surrogates for the in vivo situation while at the same time keeping test methods simple enough to allow the generation of reproducible and predictive results.

Although not addressing metal releases after exposure to gastric fluid, the study of Herting and colleagues has been included below to illustrate that metal release from alloys exposed to various biological fluids may be different than that of their pure metal constituents (Figure 1). This is particularly pronounced for alloys with passive surface oxides or with superior barrier properties, such as stainless steels (Herting et al., 2005; Herting et al., 2008a; Hedberg et al., 2011; Hedberg et al., 2013). Similar outcomes have also been demonstrated for other fluids and other engineering alloys with non-passive surface oxides (Bertling et al., 2006; Goidanich et al. 2008; Mazinanian et al., 2013; Hedberg et al., 2013). Figure 1B shows that the amount of nickel released from austenitic stainless steels is neither proportional to the bulk alloy

composition nor to the composition of the surface oxide layer where nickel is not present (Odnevall Wallinder et al., 2006; Hedberg et al., 2013; Stockmann-Juvala et al., 2013).

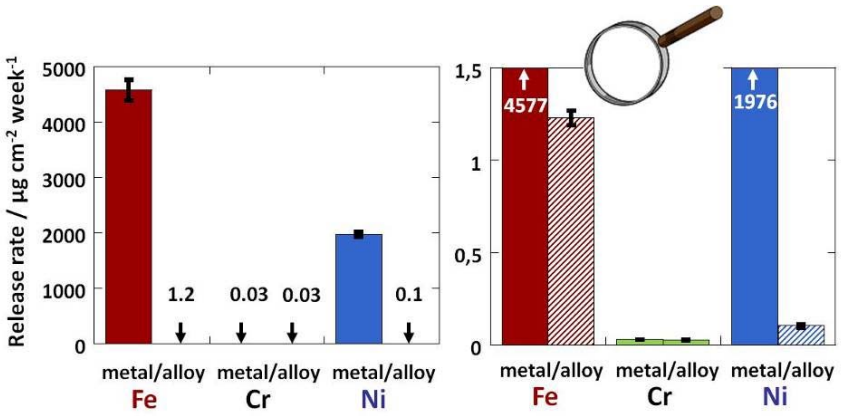

Figure 1A. Data generated from bioaccessibility studies in a synthetic lung fluid (artificial lysosomal fluid (ALF), pH 4.5, 37 °C) after 168 h of immersion. The passive surface oxide on stainless steel (AISI 316L, above identified as alloy) acts as an efficient barrier hindering the release of iron and nickel compared to non-passive oxides on the pure iron and nickel metals. Similar release rates from pure chromium and stainless steel are observed, resulting from similarities in surface oxide composition (Herting et al., 2008b).

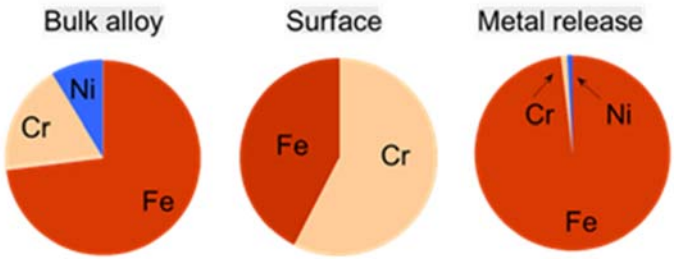

Figure 1B. Illustrates that the extent of metal release (right) cannot be assessed from either the bulk (left) or the surface composition (center) for stainless steel (AISI 316L) exposed at non-sheltered urban atmospheric conditions during four years (Odnevall Wallinder et al., 2006). Chromium, Cr; Iron, Fe; Nickel, Ni.

To conclude,

- Alloys are unique materials of disparate intrinsic properties compared to their individual metal constituents.
- The extent of corrosion/oxidation and degree of metal release is strongly related to the characteristics and surface composition of the metal/alloy and to the formation rate of oxides and corrosion products, which in turn are related to prevailing environmental conditions and alloy properties and characteristics
- Central to the issue of hazard identification and the determination of the toxicity of alloys is its rate of transformation into potentially bioavailable ionic and other metal-bearing species in the relevant environmental or physiological media.
- Similar to metals and metal compounds, speciation of released metal ions in solution is of paramount importance for hazard assessment of alloys.

2. HAZARD IDENTIFICATION AND CLASSIFICATION

Although the GHS defines an alloy as "a metallic material, homogeneous on a macroscopic scale, consisting of two or more elements so combined that they cannot be readily separated by mechanical means," alloys are explicitly considered to be mixtures for the purpose of hazard classification under GHS (UN, 2013) and EU REACH. However, alloys may not be simple mixtures of the elements from which they are composed. Rather, they may have unique physical, mechanical and chemical properties that affect the bioavailability of its individual metal constituents. As such, the **bioaccessible concentration** of a metal in an alloy is typically a better predictor of toxicity than is the metal's nominal concentration in the alloy. Recognizing the possibility that some types of mixtures may react differently than the sum of their parts, EU REACH guidance provided a distinction between simple mixtures, called "preparations," and "special preparations," such as alloys, whereby the inclusion in a matrix shall be considered" (EC, 2007).

Box 2: Definition of Bioaccessible Concentration

The **Bioaccessible Concentration** (BC) of a constituent element (usually a metal) in an alloy is based on the relative ion release from the alloy compared to the ion release from the reference substance (usually the pure metal, although a compound of the metal can be used in exceptional cases, see One-pager 1). The BC is the actual concentration of the bioaccessible metal in the alloy, based on results from bioelution tests.

The BC can be calculated as follows:
BC = Nominal concentration of metal in alloy x relative metal ion bioaccessibility (i.e., bioaccessibility of metal ion from metal in alloy compared to bioaccessibility of metal ion from pure metal reference material)

whereas relative bioaccessibility:

$$\frac{\text{(mg metal ion released from alloy/ g metal present in each g of alloy tested)}}{\text{(mg metal ion released from reference material/g reference material tested)}}$$

Alternatively the Bioaccessible Concentration can be directly calculated as follows:

$$BC = \frac{\text{(mg metal ion released from alloy/g alloy tested)}}{\text{(mg metal ion released from reference material/g reference material tested)}}$$

Example: alloy with 10% metal X. Bioelution test results (e.g. 2 hours, gastric fluid): 1 mg metal X ion release per g alloy; 30 mg metal X ion per gram pure metal X reference material.
The %BC calculated in the two different ways is shown below:

1) BC=10% nominal concentration x relative bioaccessibility [(1 mg metal ion X/0.1 g metal X per g alloy tested)/(30 mg ion metal X/g metal X tested)]= 10% x 0.33 = 3.3%

2) BC= ion released from alloy (1 mg metal X ion ion/g alloy tested)/ metal X ion released from reference material (30 mg metal X ion/g metal X tested) = 3.3%

An overall framework for human health hazard identification and classification of alloys is proposed in this section. This framework was developed based on adaptation of the following: mixture classification guidelines identified in GHS and EU CLP, the concepts of special preparations under EU REACH, grouping, and read-across formulated as part of EU REACH/OECD and other programmes, and the available scientific

data on alloys and metals (including the concept of bioaccessible concentration). A conceptual overview of the framework is presented in Figure 2 with each approach further discussed in subsequent subsections.

The first step in the hazard identification and classification process for alloys involves the collection and evaluation of available data on the alloy, its constituents and its composition. Depending on the sufficiency and quality of these data, four possible approaches can then be employed for each route of exposure and endpoint as deemed appropriate. The **Alloy-specific Approach** (Section 2.1)[1] should be used when toxicity data specific to the alloy in question are available and can be directly compared to the criteria for hazard classification. The **Default Approach** (Section 2.2) will be used when few or no alloy-specific data are available. It uses the nominal concentration of metal constituents in the alloy. The **Bioelution-Supported Bridging Approach** (Section 2.3) is used when sufficient data are available to group target[2] alloys with other similar alloys for which the hazard classification is possible and read-across (using a weight of evidence that includes bioaccessibility data) can be justified. Alternatively, if bridging is not possible but sufficient and reliable alloy-specific bioaccessibility data are available, those data can be used in a **Bioaccessible Concentration Approach** (Section 2.4). In order to use the bioelution-based approaches described here, appropriate bioaccessibility data must be available.

While the overall framework is applicable to all endpoints and routes of exposure, the illustrations (Box 4 and Box 5 of the Bioaccessible Concentration approach (Section 2.4) are focused on the oral route of exposure and systemic effects.

[1] Article 6.3 CLP: Identification and examination of available information on Mixtures/ 3. For the evaluation of mixtures pursuant to Chapter 2 of this Title in relation to the 'germ cell mutagenicity', 'carcinogenicity' and 'reproductive toxicity' hazard classes referred to in sections 3.5.3.1, 3.6.3.1 and 3.7.3.1 of Annex I, the manufacturer, importer or downstream user shall only use the relevant available information referred to in paragraph 1 for the substances in the mixture.

[2] A "target" alloy is one for which specific toxicity data are not available and are being estimated from a "source" alloy for which hazard classification is possible.

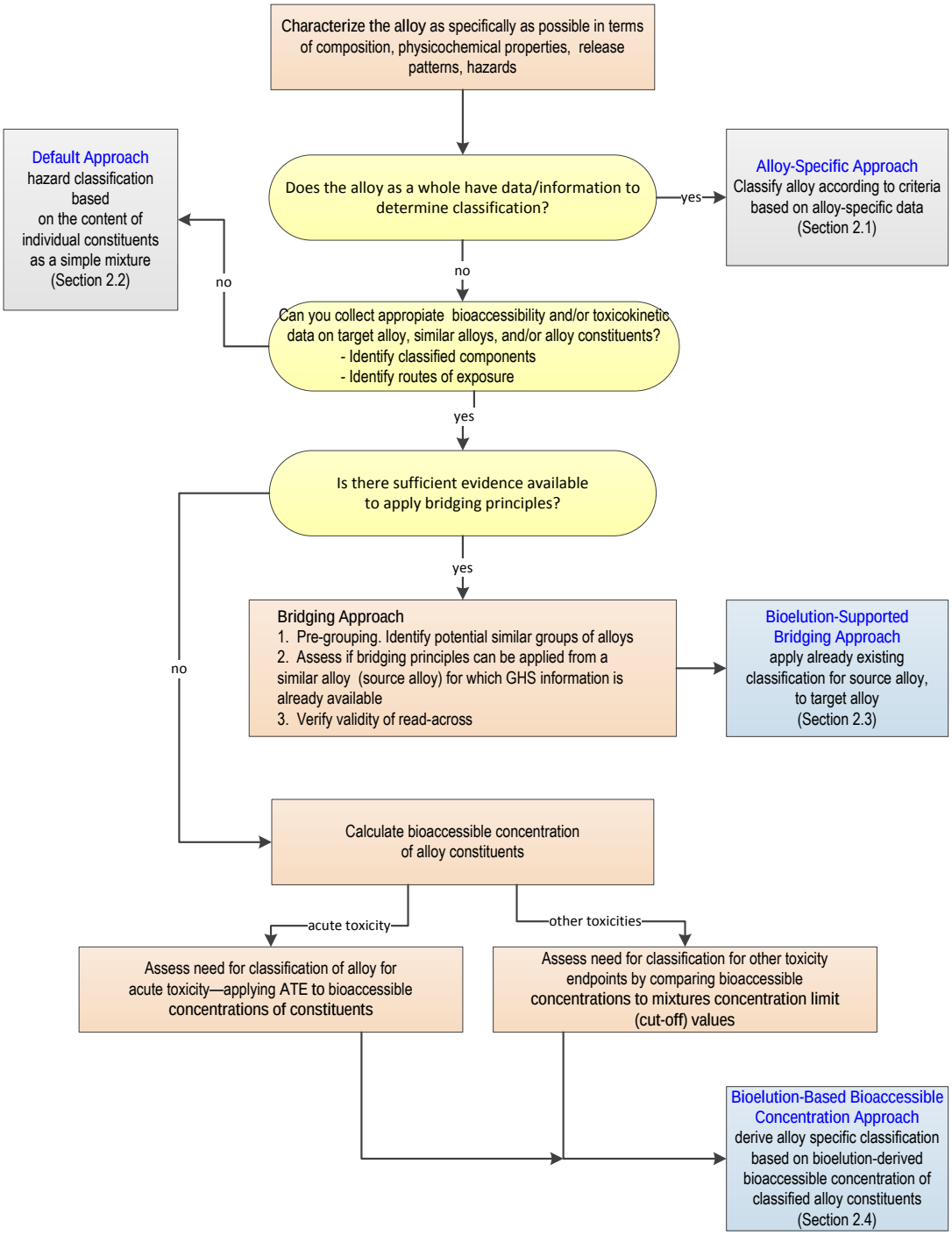

Figure 2. Conceptual tiered approach for human hazard identification and classification of alloys

2.1 Alloy-specific approach

There are some instances where alloy-specific toxicity data exist for at least one health endpoint. For example, nickel-containing alloys in massive forms can be tested using EN1811 and based on the results from this test (i.e., rate of nickel ion release) their classification as dermal sensitizers can be determined, independent of their nickel content. These results have been corroborated by patch-testing human volunteers with alloy discs. There is one example where the toxicity of an alloy powder (SS316L) was tested in rats in a 28-day inhalation study. The data generated in this study can be used to decide whether a classification as STOT-RE is needed for SS316L (Stockmann-Juvala et al., 2013). No current example exists for alloy-specific effects occurring after oral exposure.

Furthermore, for the evaluation of mixtures in relation to the 'germ cell mutagenicity', 'carcinogenicity' and 'reproductive toxicity' hazard classes referred to in sections 3.5.3.1, 3.6.3.1 and 3.7.3.1 of Annex I of the CLP, the manufacturer, importer or downstream user shall only use the relevant available information for the substances in the mixture, so this approach cannot be used for CMR endpoints.

2.2 Default CLP approach

Under the default approach to hazard identification and classification, an alloy would be treated as a simple mixture (i.e., a "mixture" under EU REACH, GHS and EU CLP). Classification of the alloy is based on the hazard classifications of its individual constituents and the percent content of each constituent in the alloy, following the appropriate approaches identified in EU CLP[3] or GHS[4]: the additivity formula for acute toxicity and the cut-off concentration/limit approach for other toxicological endpoints (including CMR). The default approach assumes each of the alloy constituents elements will be 100% bioaccessible. These approaches are analogous to those described in Box 4, Figure 3 and Box 5, Figure 4, respectively.

The default approach should be selected when the following criteria apply:

- inadequate and/or insufficient data available to indicate that toxicity of the alloy differs from that which is predicted by the content of its constituent metals
- inadequate data available indicating that metal release rates from the alloy differ from the release rates from the reference material under real or simulated physiological conditions
- inadequate and/or insufficient data available to apply the bioelution-supported bridging approach (described in the next section)
- it is impractical to generate new toxicity or bioaccessibility data

[3] See EU CLP sections 3.1.3, 3.2.3, 3.3.3, 3.4.3, 3.5.3, 3.6.3, 3.7.3, 3.8.3 and 3.9.3 for endpoint-specific guidance. Guidance on the Application of the CLP Criteria. Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.0, November 2013, European Chemicals Agency, http://echa.europa.eu/.

[4] See GHS sections 3.1.3, 3.2.3, 3.3.3, 3.4.3, 3.5.3, 3.6.3, 3.7.3, 3.8.3 and 3.9.3 for endpoint-specific guidance. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), 2013 fifth revised edition Part 3. Human Health hazards, United Nations, http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev05/English/03e_part3.pdf.

2.3 Bioelution supported bridging approach

In accordance with GHS/EU CLP guidelines for classification of mixtures, the use of **bridging** principles should be considered for the classification of alloys for which toxicology data are not available. This approach can be used to group target alloys with other similar alloys for classification (based on alloy-specific data) where sufficient data on alloy characteristics (e.g., metal bioaccessibility and physico-chemical properties, chemical composition, technical performance…,) are available. This ensures that the classification process uses the available alloy data to the greatest extent possible without relying on additional animal testing when it is unnecessary.

Box 3: Definition of Bridging

Bridging has been defined by EU CLP as follows: If sufficient information is available on similar tested mixtures, including relevant ingredients of the mixtures, it is possible to determine the hazardous properties of an untested mixture by applying certain rules known as 'bridging principles'. Those rules allow characterisation of the hazards of the mixture without performing tests on it, but rather by building on the available information on similar tested mixtures. Where no or inadequate test data are available for the mixture itself, manufacturers, importers and downstream users should therefore follow the bridging principles to ensure adequate comparability of results of the classification of such mixtures.

Bioaccessibility data are part of the evidence that allow read-across and grouping to support the bridging approach for hazard classification of alloy. Read-across strategies for metals using *in vitro* bioaccessibility data have been developed by various research entities for EU REACH purposes; with some modifications, this can also be used for alloy. The main principle driving this approach is that bridging can be used if hazard classifications exist for a "source" alloy *and s*ufficient data exist to demonstrate a "target" alloy has similar exposure behaviour (e.g., release rate of metals) relative to the source alloy. The bioelution-supported bridging approach for the oral route of exposure is outlined below:

- **Step 1**: Derive metal release data (i.e., bioaccessibility data) for equivalent amounts of the source alloy and the target alloy using the appropriate bioelution protocols and artificial biological fluids relevant to the oral route of exposure.

- **Step 2**: Develop a matrix listing data on bioaccessibility, additional physicochemical properties (e.g., surface properties), health effects, hazard classifications, and other relevant properties for both the source alloy (with their metal constituents) and the target alloy. For example, many alloys are already grouped in numerous national and international standards (e.g., AFNOR, AISI, DIN, ASTM, JISI, UNS, etc.) based on their chemical composition. In addition, they can also be grouped with respect to their technical performance (e.g., Council of Europe Guidelines on metals and alloys for food contact applications). Such information are part of the weight-of-evidence approach. Use the relationship between bioaccessibility and health effects in the source alloys to read-across to, or predict, the unknown health effects information for the target alloy, based on similarities in bioaccessibility and other factors, using a weight of evidence approach.

- **Step 3**: Use relevant and applicable information to verify that the assumptions behind the read-across paradigm are valid. This may require generation of additional *in vitro* or *in vivo* toxicological or toxicokinetic data in one or more alloys.

As discussed in Step 2 above, bridging between alloys should be performed based on the source alloys, with known hazard profile, as a whole. The release of all toxic constituents (i.e., those with existing hazard classification(s)) from the source alloy should be compared to the release of the same constituents from the target alloy when tested under the same, relevant conditions. If the releases of all toxic metals from the target alloy are the same or lower than those of the source alloy, then the hazard classifications of the source alloy can be read-across to the target alloy. If the release of one or more hazardous constituents is higher from the target alloy, then it cannot be considered to have equal or lesser toxicity, and therefore bridging would not be appropriate.

2.4 Bioaccessible Concentration (BC) approach

When bridging is not possible but sufficient and reliable alloy-specific bioaccessibility data are available, those data can be used in a Bioaccessible concentration approach. This approach focuses on tools that could potentially improve hazard identification and classification for alloys using relative bioaccessibility data. To do so, the bioaccessible concentration of each hazardous constituent in the alloy is calculated and compared to the relevant criteria for classification (e.g., calculation of acute toxicity estimate (ATE) for acute toxicity or comparing to mixtures cut-off values for other hazard classifications).

Specifically, *in vitro* methods simulating metal release in biological fluids (i.e., bioelution tests) are considered as a means of calculating the bioaccessible concentration of the toxic metal ion in alloys to be used for refining hazard identification for alloys. There are a few examples whereas the bioaccessible concentration of a metal in an alloy has been demonstrated to be a better predictor of in vivo toxicity than its content.

GHS/EU CLP classification using the bioelution-based bioaccessible concentration approach proceeds in one of two ways depending on the endpoint:

1. For acute toxicity, classification is based on acute toxicity values (i.e., LD_{50} or LC_{50}) for the constituents incorporated into an additivity formula. Application of this procedure to alloys is described in Box 4 and Figure 3.
2. For all other health endpoints, classification is based on cut-off values/concentration limits for mixtures.[5] Application of this procedure to the bioaccessible concentration of alloys is described in Box 5 and Figure 4.

Both these procedures are analogous to those applied using the default approach (section 2.2). As mentioned before, whenever bioaccessibility data is used a surrogate for bioavailability, some level of verification is needed as described in Section 2.5.

Please note that this approach applies to endpoints/effects for which toxicity is related to the metal ion. In cases where factors other than the released metal ions play a role (e.g., some local effects), other approaches (e.g., alloy-specific testing) may be required. In the case of the oral route of exposure, the situation is simpler, as it is the soluble metal ion that will be responsible for the systemic toxicity effects.

[5] The health endpoints to be assessed using cut-off values/concentration limits are: eye damage/irritation, respiratory/skin sensitization, germ cell mutagenicity, carcinogenicity, reproductive toxicity, single exposure target organ toxicity and repeated exposure target organ toxicity.

Box 4: Bioelution-based Bioaccessible Concentration (BC) approach for Acute Oral Toxicity endpoints
Use of bioaccessible concentration and adjusted ATEs

Rationale

For acute health effects, classification is based on comparison of acute toxicity estimate of the mixture (ATE_{mix}) with the acute toxicity category ranges defined under the GHS/EU CLP. ATE_{mix} is derived through the use of an additivity formula and the ATEs for the individual relevant constituents present in the alloy at >1% (nominal content). An ATE for an individual constituent is expressed as an LD50. The Bioaccessible Concentration approach differs from the standard approach for simple mixtures in that bioaccessibility of the alloy constituents (e.g. metal ion from metal and metalloids), instead of their respective content is taken into account to derive an adjusted ATE_{mix}.

Classification Procedure

1. Derive bioaccessibility data for target alloy and the reference material. The reference material should be as much as possible chemically similar to the form of the metal present in the tested material (alloy) and have a well-documented hazard profile (see also One-pager 2). In selecting which constituent metals to focus on, consider the relative toxicity of metal constituents of the alloy and their existing hazard classifications. In addition, the form (e.g. powder, massive) of the reference material and the alloy need to be considered
2. Determine the bioaccessible concentration of the metal in the alloy using any one of the two calculation methods described in Box 2.
3. Because this approach is applicable only to acute toxicity endpoints, the toxicity endpoint of interest will depend on the endpoints identified in the GHS for acute oral toxicity classification (e.g. LD50). This procedure must be carried out for all potentially classifiable constituents (i.e. metals, metalloids) from which the alloy is composed.
4. If no constituents have an estimated toxicity value below the upper limit of classification, no classification is necessary. If only one constituent has an estimated toxicity value below the upper limit of classification, classify based on that constituent. If more than one constituent has an estimated toxicity value below the upper limit of classification, use additivity formula described in the GHS (UN 2007) to classify.

Additivity formula (cf. CLP and GHS). Calculate ATEmix using the additivity formula and acute oral toxicity data for all ingredients of the alloy that have an ATE at or below the highest toxicity value for which classification would be required for a particular exposure route (e.g., for oral exposure, any ingredient with an LD50 ≤ 5,000 mg/kg).

$$\frac{100}{ATE_{mix}} = \sum_{n} \frac{C_i}{ATE_i}$$

Where: Ci = bioaccessible concentration of ingredient i in the alloy (% w/w)
ATEi = acute toxicity estimate for ingredient i

Classify the alloy based on the acute oral toxicity category in which the ATE_{mix} falls. For oral toxicity the following category cut-offs apply (mg/kg bw):

0	<	Category 1	≤	5
5	<	Category 2	≤	50
50	<	Category 3	≤	300
300	<	Category 4	≤	2,000
2,000	<	Category 5	≤	5,000

For acute inhalation and dermal toxicity, the approach would be similar.

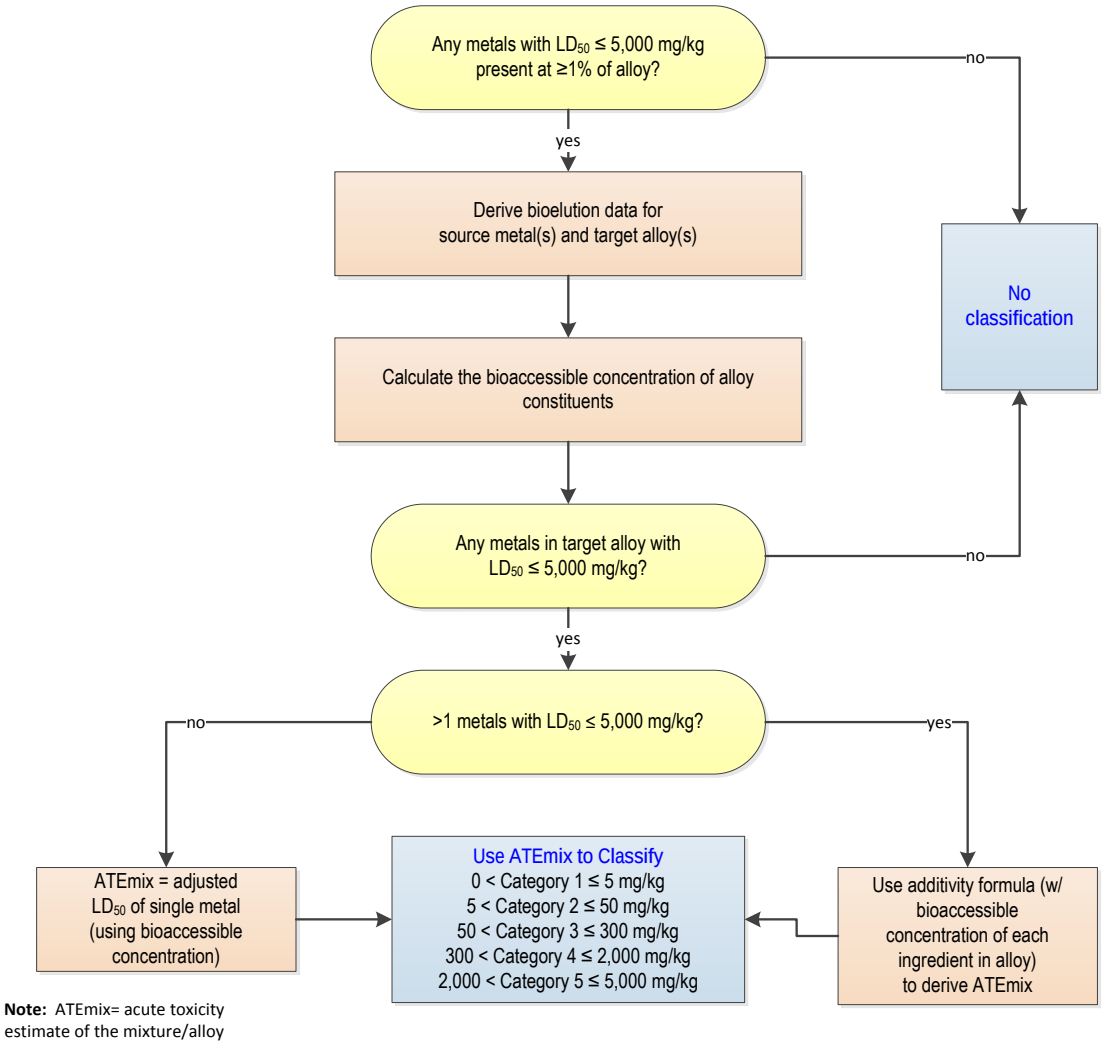

Figure 3. Classification strategy for** alloy **acute oral toxicity based on the bioaccessible concentration approach. In all cases, if range is identified for the LD_{50} value (e.g., 2,000-5,000 mg/kg), the most conservative value should be used

A hypothetical example utilizing the bioaccessible concentration approach for determining the hazard classification for acute toxicity via the oral route is provided below:

The oral LD_{50} values of the three constituent metals of alloy XYZ, to be used as the ATEs, are 7,000 mg/kg, 1,000 mg/kg, and 40 mg/kg for X, Y, and Z, respectively. The content (i.e., nominal concentration) of the respective metals in the alloy is X: 60%; Y: 36%; Z: 4% (see table below).

Bioelution testing gives the following ion releases from the metals in the alloy, as compared to the ion releases from the reference materials (relative bioaccessibility): X: 75%; Y: 50%; Z: 50%.

Based on these results, the bioaccessible concentrations of constituents X, Y, Z can now be calculated as: 45% (X), 18% (Y), and 2% (Z), respectively (table below).

Metal in alloy	Nominal concentration (metal content in alloy , %)	Relative bioaccessibility (%)	**Bioaccessible concentration** (%)
X	60	75	60*0.75 = 45
Y	36	50	36*0.50 = 18
Z	4	50	4 *0.50 = 2

Compared to the classification scheme:
One constituent (X) has an LD_{50} >5,000 mg/kg, so it is not included in the ATE_{mix} calculation.
Calculation of ATE_{mix} therefore proceeds using the corresponding ATE and bioaccessible concentration data for Y and Z as follows:

$$\frac{100}{ATE_{mix}} = \frac{C_Y}{ATE_Y} + \frac{C_Z}{ATE_Z}$$

$$= \frac{18}{1{,}000} + \frac{2}{40} = 0.068$$

$$ATE_{mix} = 1{,}470 \text{ mg/kg}$$

Based on this result, alloy XYZ would be classified as Category 4 for acute oral toxicity according to the CLP/GHS.

Box 5: Bioelution-based bioaccessible concentration approach for systemic endpoints other than acute oral toxicity
Use of bioaccessible concentration with mixtures concentration limits

Rationale

For health endpoints other than acute toxicity[6], classification of mixtures (alloy) is based on defined concentration limits. In the default approach, mixtures that contain classifiable constituents(s) at a concentration above the generic or specific concentration limit receive the same classification as its most stringently classified constituent. **The bioelution-based bioaccessible concentration approach differs from the default approach in that metal bioaccessibility is taken into account to characterize the bioaccessible concentration of metal in the alloy, which is subsequently compared to the cut-off limits defined by the generic or specific concentration limit for the classifiable constituents described in Annex VI to CLP.** Classification using this approach is summarized for the oral route in Figure 4 using germ cell mutagenicity as an example.

Classification Procedure

1. Derive oral bioaccessibility data for target alloy and the reference material. When using this approach for metals, it is appropriate to consider which forms of the classified constituents (e.g. powder, massive) are the most appropriate for assessing their bioaccessibility in the alloy being evaluated. Determine if any ingredients of the alloy are classifiable and note their classification category.
2. Determine the bioaccessible concentration of the classified alloy constituent(s). For metal constituents, the bioaccessible concentration can be calculated using any one of the two calculation methods described in Box 2.
3. If the bioaccessible concentration is greater than the **cut-off concentration** limit (i.e. the generic or specific concentration limit specified in Annex VI to CLP) for a given classified constituent, then classify the alloy based on the constituent. If the bioaccessible concentration is less than the cut-off concentration limit for all classified constituents of the target alloy, no classification is necessary. This procedure must be carried out for all potentially classifiable substances (i.e., metals, metalloids) from which the alloy is composed and for all relevant systemic health endpoints. If more than one classifiable constituent is present for a given health endpoint, the alloy will always receive the classification based on the most stringently classified constituent present above the concentration limit.

As an example, for germ cell mutagenicity, a substance can receive a category 1 (1A or 1B) classification, a category 2 classification, or no classification depending on the level of evidence for mutagenicity. Under the bioaccessible concentration approach, an alloy would receive the classification of its most highly classifiable constituent based on the following bioaccessible concentration limits.

	Bioaccessible concentration limits triggering classification of an alloy **as:**	
Ingredient Classified as:	**Category 1 mutagen**	**Category 2 mutagen**
Category 1 mutagen	≥ 0.1%	–
Category 2 mutagen	–	≥1.0%

[6] The systemic health endpoints to be assessed using cut-off values/concentration limits are: germ cell mutagenicity, systemic carcinogenicity, reproductive toxicity, single exposure target organ toxicity, and repeated exposure target organ toxicity.

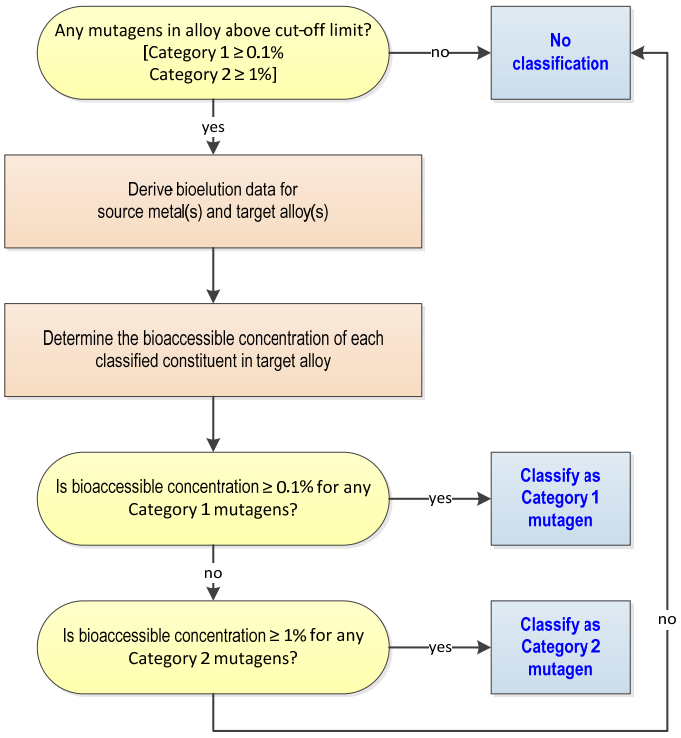

Figure 4. Example classification strategy of metal constituents for germ cell mutagenicity based on the bioelution-based bioaccessible concentration approach.

A hypothetical example utilizing the bioelution-based bioaccessible concentration approach for determining the hazard classification for germ cell mutagenicity is presented below:

The alloy has 3 constituent metals: V, W and X.
V is not a mutagen
W is a Category 2 mutagen
X is a Category 1 mutagen

The content (i.e., nominal concentration) of the respective metals in the alloy is V: 52%; W: 44%; X: 4% (see table below)

Bioelution testing gives the following ion releases from the metals in the alloy , as compared to the ion releases from the reference materials (relative bioaccessibility): V: 80%; W: 50%; X: 2%.

Based on these results, the bioaccessible concentrations of constituents V, W, X can now be calculated as: 42% (V), 22% (W), and 0.08% (X), respectively (table below).

Metal in alloy	Nominal concentration (metal content in alloy, %)	Relative bioaccessibility (%)	**Bioaccessible concentration** (%)

V	52	80	52*0.80 = 42
W	44	50	44*0.50 = 22
X	4	2	4 *0.02 = 0.08

The bioaccessible concentrations of classified constituents in alloy VWX are: 19%Y and 0.08%Z

Under the bioaccessible concentration approach, alloy VWX would be classified as a Category 2 mutagen because W is a Category 2 mutagen and is present at a bioaccessible concentration $\geq$1%. alloy VWX would not be classified as a Category 1 mutagen even though X is a Category 1 mutagen because X is present at a bioaccessible concentration <0.1%.

2.5 Verification

When any form of read-across is utilized, some level of verification is needed. Several options and approaches are available for verification and might include: 1) assembling and evaluating available toxicity data on alloys in comparison to analogous toxicity studies on the constituents for specific endpoints, 2) conducting *in vivo* bioavailability or toxicokinetic/toxicodynamic studies in representative animal species, 3) conducting *in vitro* toxicity assays or *in vivo* toxicity studies of alloy in comparison to their constituents. The above studies allow examination of the correlation between bioaccessibility and bioavailability/toxicity. Several studies have addressed this for the oral route and are detailed in One-pager 4.

3. CONCLUSIONS

This note presents a brief summary of the facts regarding the characteristics and properties of alloys and the reasons why alloys largely exhibit different properties from their individual constituents and consequently have different hazard/risk profiles. EU REACH designates alloys as a form of "special preparation" referring to the potential difference in properties from their constituents and recognising that specific assessment methods and new exposure scenarios are required. When alloy -specific toxicity data are not available for classification, we propose here a tiered approach to the human health hazard classification of alloys that benefits from the use of bioaccessibility data in relevant fluids (e.g., bridging and bioaccessible concentrations approaches to classification). When no appropriate data on the target alloy and/or the alloy constituents are available, the default mixtures (based on content of classified alloy constituents and cut-off limits for simple mixtures) approach is applied. Please note that bioaccessibility approaches for metals are appropriate for endpoints/effects for which the toxicity is related to the metal ion. Such is the case for systemic endpoints after oral exposure which is the main focus of this note. In case factors other than the released metal ions play a role (e.g., local effects), alloy-specific testing may be required.

Finally, as for any proposed approach, some level of verification is needed to ensure the requirements for correctly identifying and communicating the health hazards of alloy are met.

ANNEX 1 –ABBREVIATED VERSION OF THE BIOELUTION ROADMAP

The aim of this annex is to explain and clarify what is meant by 'bioelution', what its potential applications are, how to use bioelution results in practice and what the required work is to back up/validate its uses.

Its scope addresses metals and their inorganic compounds as well as complex materials containing metals. Complex materials containing metals are considered here to include alloys, metal-related ores and ore concentrates, and inorganic UVCBs.

Please note that the content of this note is based on experience gained with metals and their inorganic compounds and therefore should not be applied to organometallic compounds (i.e., chemical substances containing a covalent bond between carbon and the respective metal) without consideration of the fact they may behave differently.

1. Introduction:

The metal industry has great interest in minimizing the use of animal tests in regulatory compliance and in supporting the use of alternative *in vitro* methods of testing the safety of materials. Furthermore, many Regulations (e.g. EU REACH) also request that testing programmes should be conducted minimising animal testing where appropriate. There is currently extensive research ongoing to develop suitable *in vitro* methods.

These in vitro methods embrace the Three Rs concept (**R**eplace, **R**educe and **R**efine), proposed by Russell & Burch in *The Principles of Humane Experimental Technique* (1959), which launched a program for the humane treatment of laboratory animals in experimental biology. Based on the Three Rs, laws of many countries and *Directive 86/609/EEC* of the European Union that now specifically require *replacement* -, *reduction* - and *refinement alternatives* should be used wherever and whenever possible in biomedical research, testing and education.

The use of bioelution is an excellent example of a reliable alternative method to animal testing which allows compliance with strict regulatory requirements whilst minimising animal testing. Bioelution testing is used by the inorganic industry to reduce the need for animal testing in well-defined cases whilst still providing regulatory authorities and the wider audience with high quality safety dossiers that satisfy current requirements.

This document provides guidance on conducting bioelution studies and discusses their use in regulatory submissions to regulatory authorities.

Generally, **bioavailability** is defined as the extent to which a substance is taken up by an organism and is available for metabolism and interaction. The toxicity of most metals is associated to a large degree with the release of *soluble metal ions,* their uptake by the body and interaction at their target sites. Therefore, the bioavailability of most metals is defined as the extent to which the soluble metal ion can be available at to the target organ/site.

Information on bioavailability is usually obtained from toxicokinetic studies for all relevant routes of exposure and all relevant forms or physical states where the substance and/or metabolite(s) of the substance have been quantified in body fluids and/or target organs.

In situations where the bioavailability of a substance/material is not known or where it is not feasible to determine this *in vivo*, bioaccessibility may be used to estimate bioavailability. **Bioaccessibility** is defined as the fraction of a substance that dissolves under surrogate physiological conditions and therefore is "potentially available" for absorption into systemic circulation.

Bioelution refers to the *in vitro* extraction methods used to measure the degree to which a substance (e.g., metal ion) is released in artificial biological fluids. Bioelution tests are thus used to estimate a substance's bioaccessibility (in the form of metal ions), i.e. its solubility under physiological conditions.

The assumption is that the bioavailability of a metal in the various forms in which it can be present in substances and mixtures can vary considerably. Bioelution allows this to be assessed for a large number of chemical substances and mixtures.

For metal substances and complex materials containing metals, there are indeed several factors that may affect bioavailability and consequently the amount of ions that will be able to interact at a target site (e.g., physical form, the inclusion into a matrix or complex structure as in alloys, spinels, pigments, frits, glasses etc.).

Considering that it is not acceptable, from an animal welfare viewpoint, to perform *in vivo* studies for each specific material, insight into processes determining bioavailability is required to be able to predict toxicity in a weight-of-evidence approach. Bioaccessibility testing –to estimate bioavailability -has become an active research area with both individual researchers and research groups working to develop and validate bioelution protocols.

The main impetus for the development of *in vitro* bioelution protocols and use in substance/material data development is to minimise animal testing whilst generating a conservative and robust output.

The main advantages of bioelution tests to predict bioavailability can be summarized as follows:

- Reduction in animal testing
- Results from bioelution tests are reproducible
- Bioelution tests are conservative and estimate the potential bioavailability of the test substance (bioaccessibility). This will result in a protective assessment as absolute releases may overestimate the bioavailability potential *in vivo*
- Bioelution tests are inexpensive & rapid
- Bioelution tests can be tailored to provide data for **specific exposure pathways** e.g.
    - Dermal exposure: the dissolution in artificial sweat can be used to estimate the metal ion bioaccessibility in the surface layer in contact with the skin to support predictions for both sensitisation potential and systemic effects following dermal uptake. The release of potential skin sensitising metal ions in sweat has already been recognised as an intrinsic property of alloys that can drive their classification (example: Ni, referred to in Note 7 of

the CLP[7]).

- Oral route: the dissolution in various artificial gastric/intestinal/saliva fluids can be used to estimate the relative metal ion bioaccessibility to support predictions of systemic effects following oral exposure
- Inhalation route: for this particular route, given its complexity, a more mechanistic approach should be applied, where, in addition to inhalability and particle deposition, data from dissolution in simulated lung/lysosomal fluids can provide support for predictions of systemic inhalation effects. For local effects, factors other than the concentration of the ion at the target site may be important in determining toxicity in the lung (e.g. particle effect, lung overload, redox reactions, oxidative stress, change in pH). The fractional release of metal ions will be a contributing factor to the hazard properties of alloy particles for respiratory effects. Although bioelution tests may give a first estimate of the 'persistence'/dissolution of the substance at the lung level, the outcomes should not be used in isolation to predict toxicity.

The potential applications of bioelution testing for metals are hazard identification of materials, hazard classification, read-across & bridging and grouping of substances and complex materials. For example, *in vitro* bioaccessibility has also been used by US EPA to predict relative bioavailability of lead from soil for gastrointestinal absorption.

Bioelution results should always be used in a conservative, weight-of-evidence approach as there are limitations to the test and applicability (see section 2). In order to be acceptable to regulatory authorities and to give confidence to the user of the metal, substance and complex material, it is imperative that all regulatory submissions and applications contain high quality, reproducible data and that the scope is well defined.

2. Availability and status of bioelution methods

2.1 Existing regulatory guidelines on bioelution

Bioelution is not a 'new' concept and in some regulatory arenas it is already well-established in assessing the bioavailability of metals in environmental matrices and articles.
For example, the following bioelution methods have been formalised as standards for product testing:

EN 71.3 and ASTM F-963	Methods for determining toy safety, which specifies requirements for the migration of metals from toy materials
EN 1811	Nickel release from consumer articles intended for prolonged and direct skin contact
ASTM D-5517	Extractability of metals from art materials
BARGE	The Bioaccessibility Research Group of Europe (BARGE) studies human bioaccessibility of priority contaminants in soils such as arsenic, lead and cadmium via the gastrointestinal tract

Guidance on how to conduct bioelution tests is also available from several regulatory authorities (e.g., U.S. EPA 2004; RIVM 2005, 2006).

[7] Alloys containing nickel are classified for skin sensitisation when the release rate of 0,5 µg Ni/cm²/week, as measured by the European Standard reference test method EN 1811, is exceeded (CLP)

2.2 Pivotal considerations in developing bioelution methods

A number of guidelines outlining how to carry out bioelution testing already exist (see above). It is recognised that clarity and reproducibility of the methodology are key aspects to allow the application of bioelution testing. The development of reproducible tests, utilising internationally recognised protocols is pivotal in the following aspects:

1. Choice and composition of artificial fluids should accurately reflect the route of exposure to a metal substance/matrix.
2. Research has demonstrated that there are several parameters in the bioelution settings that can have significant influence on the dissolution kinetics of the tested materials. Examples of such parameters are: composition, ionic strength and pH of the extraction fluid, temperature, light conditions (e.g. darkness), fluid agitation rate, duration of the extraction process, loading and particle size/surface area of the tested substance and presence of chelating or complexing agents (e.g., see OECD 211).[8] Those parameters should be carefully considered in the design of the bioelution methods to ensure reproducibility and reliability, but also their influence on the interpretation of results should be taken into account. In particular it should be assured that these parameters do not differentially influence the release of metal ions from alloy and constituents so that the relative metal releases stay the same. Dissolution should not be forced or limited by test conditions that are not representative of the conditions under which the material would be exposed in an in vivo system.
3. The nature of the substance under investigation can affect the results of bioelution testing, since altering a substance can have significant effects on the outcome of the study. For example if a metal or substance is milled prior to testing, this may change the matrix properties of the test material and could result in spurious results. It is therefore important that as far as possible and feasible the test utilises the substance as it is expected to be available under the foreseeable conditions of use. For massive metals, surface area related test conditions can allow for normalization of metal ion releases by surface area and can be used to determine the bioaccessibility of metals from different forms of materials.
4. Release results can be expressed in terms of either the bioaccessibility of the metal in e.g. mg metal ion/litre, mg metal ion/g sample, mg metal ion/g metal in sample or as rates (mg metal ion/litre/hour) or corrected by surface area when relevant. Indeed, for metals and some alloys, the ion release results from surface reactions, including oxidation, leading to dissolution.

2.3 General principles in bioelution testing with regard to their applicability and emphasis on the oral route and systemic effects

Bioelution methods are expected to provide a conservative approximation of the bioavailable fraction of metals and other substances that may be released from materials under physiological conditions. As such, the outcomes should be used as part of a **weight-of-evidence** approach to assess the bioavailable fraction, or potentially absorbable dose following human exposures. Other components of such a weight-of-evidence approach would include information regarding the physical size and form of the materials, toxicokinetics, the pattern of exposures (with regard to route, frequency, and duration), and other details regarding the physical and chemical nature of the specific material of interest (e.g. surface area, precipitation, adsorption, chelation, speciation, possible effect of counter-ion).

[8] Experience has shown that properties of the medium may affect the outcomes when testing metals and metal compounds. This is e.g. referred to in OECD 211.

There is a need for biological verification of bioelution data and/or supporting data, e.g. verification using available animal toxicokinetics or toxicity data. Simple extraction methods may not accurately predict the bioavailability of substances in all cases, because biological processes can be complex. In the case of some metals, the valence of the tested metal has to be established as the metal may exist in several valence states, which in turn have considerable toxicological significance (for example in the case of e.g. chromium and vanadium).

The US EPA has conducted a detailed validation programme for an in vitro method to predict the relative bioavailability of lead from ingested soils (US EPA 2005a). The BARGE group has data from similar studies with other metals. However these data may not be applicable to other exposure routes and it would be preferable to obtain specific data for each metal or material and for each route of exposure. As the bioelution method is expected to complement or even in defined cases to replace an animal study, it is important that the resulting data are conservative, reliable and reproducible to give confidence in the results.

Bioelution should reflect "conditions of exposure and absorption". *In vivo*, dissolution and absorption of a substance may occur in several sites, even for a particular route of exposure. For example, bioelution following oral exposure may require testing under the simulated physiological conditions of the saliva and/or gastric and/or intestinal components of the gastrointestinal tract. Integrated bioelution tests (saliva + gastric + intestinal) have been developed/validated for oral absorption of metals from soils (BARGE). In other cases, gastric fluid alone may be predictive of bioavailability.

For the proposed applications outlined here below, it is important to recognise that the bioelution method is a **comparative approach**. In all cases, the test substance or material is compared to at least one reference substance or material. **The reference substance(s) or material(s) is (are) usually chosen on a case-by-case basis. Each reference should be clearly identified and the rationale for its selection recorded.** This issue is further detailed in One-pager 2.

2.4 Current developments and proposed future work by industry

A bioelution testing programme aimed at performing an inter laboratory comparison exercise of established test methods (including one based on the ASTM D-5517) has been led by Eurometaux and a number of metal associations/commodities. The outcomes are published in Henderson et al. 2014.

Henderson et al. 2014-main.pdf

A Standard Operating Procedure (SOP) integrating the main learning lessons of the exercise is currently being improved by industry and annexed to this note, together with One-Pagers addressing specific aspects related to testing, i.e. One-pager 3 on representativity of parameters selected for gastric fluids testing and One-pager 5 on literature data on gastric/intestinal sequential versus parallel testing.

To gain wider acceptance of these in vitro methods it is recognised by Eurometaux that these methods/guidelines/SOP have to become internationally accepted guidelines by OECD. Eurometaux is involved in initiating discussions at OECD and welcomes the participation of an OECD country in promoting these methods within the OECD chemical program. A draft SPSF aimed at developing a Guidance Note for

the generation and use of oral bioelution test data is included in the package.

Acceptance of these methods by regulatory authorities is indeed crucial to the continued use of these methods to minimise animal testing. Recently, several bioelution results have been submitted to the regulatory authorities under the REACH programme in the EU. Examples of these are:

1. The grouping of cobalt, molybdenum and vanadium substances for registration and testing proposals under REACH
2. The classification of complex materials (e.g. UVCBs) under the CLP regulations in the EU
3. The REACH restriction on lead compounds in consumer articles that can be mouthed by children (saliva testing) (see also Urrestarazu et al., 2014)

Note: examples are described in more detail in the Appendix to the full EM Bioelution Roadmap. ***This is intended to be a living document: that Eurometaux will keep up to date with further examples and feedback from regulatory authorities as they become available.***

2.5 Limitations

As with all test methods there are limitations on how the bioelution results can be used in the evaluation of a test material. The bioelution method is usually employed as a comparative test, where toxicity data are already available on the reference substance(s) or material(s).
Some routes of exposure have been more closely examined and verification is already available, as is the case for the oral route. However, care has to be taken when evaluating other routes of exposure (e.g. inhalation) where the solubilisation of a substance or material may only be one factor in predicting toxicity, whereas other factors may play an equal or even a more significant role.
The need for verification and reproducibility is key in this type of study especially when assessing systemic toxicity. It is important to accept that bioelution is only part of a submission, where a weight of evidence argument is presented and all sections of this argument have to be clearly described and discussed.

Where there is a valence issue for a particular metal (e.g. chromium and vanadium) it is important that the most relevant ion is considered in the weight of evidence discussion.
It is also important to consider the counter-ion present in some instances especially if this could be considered biologically active.

3. The main applications of bioelution for the metal industry

Bioelution can be used as a tool to measure bioaccessibility and provide an estimate of bioavailability, which itself has a number of potential applications. Bioavailability considerations may indeed influence hazard classification, e.g. by refining default classifications of a material with what is “bioavailable” and/or by justifying derogation for lack of bioavailability.

Bioavailability considerations are also part of the weight of evidence approach applied to read-across (extrapolation of known data from one substance to another substance based on the assumption that the two substances will cause similar biological responses) and bridging (when information on a mixture is not available, data on similar tested mixtures and on the ingredient substances are used instead).

Bioavailability can also be used as a tool to establish categories of substances, mixtures and complex materials for e.g. hazard identification purposes and/or testing proposals.

How to use bioelution data for hazard identification and classification of alloys is further described in section 2 of this note.

It is stressed here, as also included in the full bioelution roadmap, that bioelution methods are expected to provide a conservative approximation of the bioavailable fraction of metals and other substances that may be released from materials under physiological conditions. As such, the outcomes should be used as part of a **weight-of-evidence** approach to assess the bioavailable fraction, or potentially absorbable dose following human exposures. Other components of such a weight-of-evidence approach would include information regarding the physical size and form of the materials, toxicokinetics, the pattern of exposures (with regard to route, frequency, and duration), and other details regarding the physical and chemical nature of the specific material of interest (e.g. surface area, precipitation, adsorption, chelation, speciation, possible effect of counter-ion).

For the proposed applications outlined in this roadmap, it is important to recognise that the bioelution method is a **comparative approach**. In all cases, the test substance or material is compared to at least one reference substance or material. Each reference should be clearly identified and the rationale for its selection recorded.

KEY TERMINOLOGY AND ABBREVIATIONS

ATE	Acute Toxicity Estimate. Used in GHS/CLP classification to characterize the acute toxicity of a substance or ingredient in a mixture. The ATE is derived using the LD_{50} (oral and dermal exposure), LC_{50} (inhalation exposure), or the conversion values in Table 3.1.2 of GHS (UN 2013), CLP 2008 when a range of toxicity values is available or for classification of a mixture
Bioaccessibility	The fraction of a substance that dissolves under surrogate physiological conditions and therefore is potentially available for absorption into systemic circulation (systemic effects) or for interaction at port of entry sites (local effects)
Bioaccessible concentration	The bioaccessible concentration of a constituent substance (usually a metal) in the alloy; is based on the relative metal release from the alloy compared to the metal release from the pure metal
Bioavailability	The extent to which a substance is taken up by an organism and is available for metabolism and interaction
Bioelution	The *in vitro* extraction methods used to measure the degree to which a substance (e.g., metal ion) is released in artificial biological fluids
Bridging	"If sufficient information is available on similar tested mixtures, including relevant ingredients of the mixtures, it is possible to determine the hazardous properties of an untested mixture by applying certain rules known as 'bridging principles'. Those rules allow characterisation of the hazards of the mixture without performing tests on it, but rather by building on the available information on similar tested mixtures. Where no or inadequate test data are available for the mixture itself, manufacturers, importers and downstream users should therefore follow the bridging principles to ensure adequate comparability of results of the classification of such mixtures." (EU CLP, 2008)
CLP (EU CLP)	Classification, Packaging and Labelling of Substances And Mixtures Regulation
GHS (UN GHS)	Globally Harmonized System (of classification and labelling of chemicals)
Hazard category	The division of criteria within each hazard class that compares severity within a hazard class but not between different hazard classes
HERAG	Health Risk Assessment Guidance (for Metals)
Mixture	Defined under GHS as a mixture or solution composed of two or more substances which do not react; synonymous with the term *preparation* used under EU REACH
REACH (EU REACH)	Registration, Evaluation, Authorisation of Chemicals

Read-across	The process by which health/hazard information for one chemical is used to predict the same health/hazard endpoint for another chemical that is considered to be similar in some way
Source alloy	An alloy for which health/hazard information is known. The source alloy is used to extrapolate information to a target alloy about which less is known.
Special preparation	A complex mixture where the properties of the constituent substances are modulated by their incorporation in a chemical matrix. "Alloys are preparations under REACH, albeit special ones where the properties of the preparation do not always simply match the properties of the components" (EC 2007)
Substance	"Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition." (UN 2013)
Target alloy	An alloy for which endpoint information is not available and thus this information is being estimated from another source alloy or from its constituent metals.

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One-pager 1: Comparison of default and proposed approach including conservatism

Questions: Is the approach proposed by industry, referring to a bioaccessible concentration conservative enough versus the default CLP mixtures approach? Does it cover situations where the consideration of the matrix effect results in higher bioaccessible concentrations than nominal concentrations? Is there an equivalent to the environmental classification chronic 4 'safeguard'?

The presentation made at the 27 April meeting included a comparison between the two approaches for the classification of alloys, illustrating that the proposed approach is fully aligned with the CLP default mixtures approach but considers in addition a primary mechanism of action of the metal/metal containing materials (i.e. the ion is responsible for the toxicity) and bioavailability, as well as important factors impacting it (e.g. matrix effect).

The starting point for classification is typically the composition of the mixture. However, it is known that metal ions are responsible for exerting toxic effects and it is known that there are materials whose physical form, inclusion in a matrix or in a complex form can have an impact on the release of the metal ions and therefore on their bioavailability.

The proposed classification approach is developed *as a refinement step* for alloys[9], as it specifically accounts for the properties of these mixtures.

In the table below the similarity and specificity of the two approaches are summarized, highlighting how the bioaccessible concentration (BC) approach can be seen as a refinement tier in the application of CLP metal mixture approach.

[9] The term 'alloys' is used in line with the UN GHS definition but the concepts developed in the note/one-pagers also apply to "intermetallic mixtures equivalent to alloys"

Human Health hazard endpoints	CLP Default mixtures approach	Bioaccessible concentration (BC) approach
Description of the approach to classification of alloys	Concentration of classified constituent in mixture is compared to cut-off concentration values for each health endpoint (relevant to oral, inhalation and dermal routes of exposure)	Metal ion release-based BC of classified constituent in alloys is compared to cut-off concentration values for each health endpoint (relevant to oral, inhalation and dermal routes of exposure)*
Does approach consider toxicity to be related to metal ion?	**NO**. Toxicity is assumed to be related to concentration of metal in mixture	**YES,** directly for oral route and systemic effects, contributes to local effects by inhalation-dermal
What is the reference substance?	Classified constituent metal (or metalloid) present in alloy	Classified constituent metal (or metalloid) present in alloy
Is there a reference value? (cut-off) Is it substance-specific?	**YES.** Toxicity classification is related to metal content and the default GCL or SCL (substance-specific) in some cases	**YES**. Toxicity classification is related to bioaccessible metal content in alloy and the default GCL or SCL (substance-specific) in some cases
Do you measure metal releases? Absolute or relative metal releases?	**NO.** No measurements of metal release are made	**YES.** Relative releases and Bioaccessible Concentration (BC, %)
Does it allow different classification of massive and powder forms of alloy?	**NO.** Only concentration matters, not the physical form	**POSSIBLY if** the BC of classified constituent is different in massive and powder forms of the alloy, powders are produced by specific industrial processes, and they are not generated from the massive form during normal handling and use
What materials does this approach apply to?	By default, all mixtures	Alloys
Does this approach capture the alloying-matrix effect?	**NO**	**YES**

With regard to **conservatism**, we believe that both approaches entail the same level of protection and in addition, a safety net approach can be proposed. This is explained here below, using as starting point an alloy with x% content of a metal.

Bioelution tests are carried out to calculate the Bioaccessible Concentration (BC) in the alloy by comparing metal ion releases from the alloy vs. metal ion releases from the reference material (pure metal in case of an alloy, see also one-pager 2 on Reference Material).

The BC is calculated as follows:

BC = Nominal concentration of metal in alloy x relative metal ion bioaccessibility (i.e., bioaccessibility of metal ion **from metal in alloy** compared to bioaccessibility of metal ion **from pure metal**)

↕

BC= % metal in alloy x relative bioaccessibility (matches definition above)

For example, for an alloy with 10% metal X, with the following bioelution test results: 1 mg metal X ion/ g alloy ; 30 mg metal X ion/g pure metal X, the Bioaccessible Concentration will be:

BC= 10% x [(1 mg metal X ion/0.1 g metal X in alloy)/ (30 mg metal X ion/g metal X)] = 10% x 0.33 = 3.3%

Depending on the type of material, we could have three scenarios: the BC can be either <x%, = x% of > x%, where x is the concentration of the metal in the alloy expressed in %:

Scenario 1: BC is <x% (the matrix effect decreases the metal bioaccessibility): in this case the BC of the metal in the alloy gets compared to the endpoint cut-off (SCL, GCL) to determine if the classification of the alloy (based on metal classification) is warranted

Scenario 2: BC = x% (there is no matrix effect and the alloy behaves as a 'simple mixture'), the BC (or the metal content) gets compared to endpoint cut-off to determine if classification is warranted

Scenario 3: If BC is > x% (the metal in the alloy shows an increase in bioaccessibility), the BC gets compared to endpoint cut-off to determine if classification of alloy (based on metal classification) or an alternative approach (see below) is warranted.

Is the BC approach a safe and conservative approach?

In Scenario 3, the BC is > x% (the metal in the alloy shows an increase in bioaccessibility). In this scenario we could have that the BC is > x% but still ≤100%. As long as the BC is ≤100%, this is a safe approach and even more conservative than basing the classification on % content, since some alloys would be classified as hazardous to health as a result of enhanced bioaccessibility of the metal ion from the alloy compared to that predicted by the % content.

Please note that when BC =100% it means that the alloy behaves as pure metal. In other words, although the content of the metal in the alloy is << 100%, the presence of other elements in the alloy enhance the release of the metal ion to the point that the alloy ends up releasing as much metal ions as if it contained 100% of the metal.

If the BC >100%, an alternative approach to classification must be adopted. This could include obtaining toxicity data on the alloy itself (whilst respecting CLP Article 6 (3)) or by application of bridging principles with other alloys that behave similarly. The classification profile for all the chemical forms of the metal could be examined. In either case, there will be the need to use a weight of evidence approach and expert

judgement in order to arrive at an appropriate classification when bioelution testing of the alloy illustrates a matrix effect that enhances the release of toxic components beyond the maximum that can be expected when using the pure metal as a reference.

For example, for an alloy containing 60% metal Y with bioelution test results: 60 mg metal Y ion/ gram alloy; 30 mg metal Y ion/g pure metal Y

BC= 60% x [(60 mg metal Y ion/0.6 g metal Y in alloy)/ (30 mg ion metal Y/g metal Y)] = 60% x 33,33 = 200%.

One-pager 2: Reference material

Question: if the BC (Bioaccessible Concentration) is calculated by comparing releases of the ion from the metal in the alloy vs. a reference material, how should the reference material be selected? What information do we need?

First of all, one should distinguish the reference material from an internal standard:

The **reference material** is a homogeneous and stable sample with respect to specific properties. The purpose of the reference material is to allow testing of a representative substance, relevant for the alloy under investigation, and that is adequately described to ensure testing reproducibility. The ion release of a particular element from the material under investigation (alloy[10]) is measured and compared to the ion release of the same element from the reference material.

The analytical standard is a testing material relevant for the specific testing procedures and which does not need to match properties of the material under investigation. The purpose of an **analytical standard** is to check the performance of the analytical procedures, e.g. whether the extraction process (and the analytical process) have worked in a proper way, to check the possible external contamination, and to ensure reproducibility of the test procedures.

Therefore, the analytical standard can be different from the material under investigation and does not even need to contain the metal under investigation. The expected outcome of the analytical standard should be known and validated.

While we do not need to know the hazard profile of an internal standard, as it only validates the laboratory test system, we need to have enough information on the reference material (see below).

We need to document and justify the choice of the reference material to which we will compare the release results from the alloy under investigation.

How?

In generic terms, the reference material should be as much as possible chemically similar to the form of the metal present in the alloy and have a well-documented hazard profile. The "best match" based on physico-chemical parameters relevant for metal bioavailability and toxicity as well as data abundance on toxicity/classification should be used as reference material for the metal form(s) present in the tested metal-containing material.

For an alloy, where the metal constituent will be under the metallic form, it is proposed to use as much as possible the pure metal as reference material (e.g., Ni metal for stainless steel alloys). In the specific cases where there is a matrix effect that increases the bioaccessible concentration of the metal in the alloy > 100%, additional information in a weight of evidence evaluation has to be considered (see One-pager 1).

In all cases, **the choice of the reference substance** is critical and needs to be scientifically justified.

[10] The term 'alloys' is used in line with the UN GHS definition but the concepts developed in the note/one-pagers also apply to "intermetallic mixtures equivalent to alloys"

Examples of phys-chem parameters relevant for bioavailability are listed below. This information is considered relevant to support and document the 'best match.'

- Form, shape and particle size: It is reminded that in line with CLP, the substances/mixtures used in the bioaccessibility tests should be in the form in which they are placed on the market or in the form in which they can reasonably be expected to be used. The physical form of solid metals and alloys can influence the bioavailability of a compound. Several forms of solids exist: massive, briquette, flasks, granular, sponges and powders. If bioelution is used in order to assess toxicity by oral route, the relevant form for exposure should be tested.
- Oxidation state/speciation: Different oxidation states give different chemical properties to a substance and to the alloy when the substance becomes a part of it. This information should be available in order to optimize the use and understanding of bioelution results, to select the most appropiate reference and to assess toxicity. In the case of alloys, the oxidation state/speciation of released metal ion should also be considered
- Surface area is an important element in the solubility / bioavailability of a substance. More and more studies are showing that in addition to particle size, one should consider surface to explain the difference of solubility and toxicity of powders. Surface is a specific zone of a substance which will react differently in the body as central zone of chemical reactions such as oxidation or reduction. These modifications should have an influence on the substance itself and are also to consider in order to understand bioelution/ bioavailability mechanisms

These aspects should be as comparable as possible for the tested material (e.g. alloy) and the reference material.

Other points will also have to be described in the test : number of samples, purity of the tested material, storage conditions to ensure integrity of the substance. Further details are provided in the Standardised Operating procedure.

One-pager 3: Representativity of the fluids

Questions: do we have evidence, literature data justifying the composition of the fluids we are proposing to use as well as the sampling time? Scope: gastric juice to be used in the context to estimate bioaccessibility after exposure via the oral route, systemic effects.

This one-pager reports the literature data we have found, supporting the **composition** of gastric juice as referred to in the Standardised Operating procedure (SOP) (also annexed), the selected **pH** of 1.2-1.5 and the **sampling time**.

a) Composition

Details on the composition of gastric fluid are provided by e.g. Comité Européen de Normalisation standard – Safety of toys (BS EN 71-3,2013) – adopted in US as ASTM D5517 (2007; Standard Method for Determining the Solubility of Metals in Art Materials. The composition of gastric fluid employed in a variety of studies (see reference list below) is generally pretty uniform with HCL acid (0.07N) and pH in 1-2 range being the most common fluid composition. In some instances pepsin, surfactants or glycine have been added to the fluids but they do not seem to affect the results (Galia et al., 1998) particularly for metals (Hillwalker et al 2014).

b) pH

The pH is generally reported as 1.5 (typical interval found: 1.2 to 1.8, with the lower pH more representative of fasting state and a pH of 1.5-1.6 being more biorelevant), for example in:

- Brandon E.F.A., Oomen A.G., Rompelberg C.J.M, Versantvoort C.H.M., Van Engelen G.M., Sips A.J.A.M. 2006. Consumer product in vitro digestion model: bioaccessibility of contaminants and its application in risk assessment. Regulatory Toxicology and Pharmacology, 44 : 161-171. **(*swallow model-fasted : pH 1.6)***
- Brattin W., Drexler J., Lowney Y., Griffin S., Diamond G., Woodbury L. (2013). An in vitro method for estimation of arsenic relative bioavailability in soil. Journal of Toxicology and Environmental Health, Part A , 76 : 458-478. ***(pH 1.5)***
- Denys S., Caboche J., Tack K., Rychen G., Wragg J., Cave M., Jondreville C. and C. Feidt (2012). In Vivo Validation of the Unified BARGE Method to Assess the Bioaccessibility of Arsenic, Antimony, Cadmium, and Lead in Soils. Environ. Sci. Technol., 46: 6252-6260 ***(pH 1.2)***
- Drexler J.W. and Brattin W.J., 2007. An in vitro procedure for estimation of lead relative bioavailability: with validation. Human and Ecological Risk Assessment, 13:383-401 ***(pH 1.5)***
- EN71.3 Safety Toys: Migration of certain Elements, method adopted by United States as ASTM D5517: A Standard Method for Determining the Solubility of Metals in Art Materials ***(pH 1.5)***
- Galia E., Nicolaides E., Hörter D., Löbenberg R., Reppas C., Dressman J.B. (1998). Evaluation of various dissolution media for predicting in vivo performance of class I and II drugs. Pharm Res., 15: 698-705 **(*pH 1.6)***
- Henderson R.G, Verougstraete V., Anderson K., Arbildua J.J., Brock T.O., Brouwers T., Cappellini D., Delbeke K., Herting G., Hixon G. , Odnevall Wallinder I., Rodriguez P.H., Van Assche F., Wilrich P.

and A.R. Oller (2014). Inter-laboratory validation of bioaccessibility testing for metals. Regulatory Toxicology and Pharmacology, 70: 170-181 **(*pH 1.5)***

- Hillwalker W.E., Anderson K.A. (2014) Bioaccessibility of metals in alloys: Evaluation of three surrogate biofluids. Environmental Pollution, 185: 52-58. ***(pH 1.2-1.5)***
- Juhasz A.L., Weber J., Smith E., Naidu R., Marschner B., Rees M., Rofe A., Kuchel T., Sansom L. (2009). Evaluation of SBRC-Gastric and SBRC-Intestinal Methods for the Prediction of In Vivo Relative Lead Bioavailability in Contaminated Soils. Environmental Science & Technology,43:4503–4509 ***(pH 1.5)***
- Juhasz A.L, Herde P., Herde C., Boland J., Smith E. (2014). Validation of the Predictinve Capabilities of the Sbrc-G in Vitro Assay for Estimating Arsenic Reative Bioavailability in Contaminated Soils. Environmetnal Science & Technology, 48: 12962-12969. ***(pH 1.5)***
- Maltby J. R., Lewis P., Martin A. Gastric fluid volume and pH in elective patients following unrestricted oral fluid until three hours before surgery (1991). Canadian Journal of Anaesthesia , 38 (4): 425 ***(pH 1.5)***
- Molina R.M., Schaider L.A., Donaghey T.C., Shine J.P., Brain J.D. (2013). Mineralogy affects geoavailability, bioaccessibility and bioavailability of zinc. Environmental Pollution, 182: 217-224 *(using the OSWER protocol for simulated gastric fluid extraction, i.e.* ***pH 1.5****)*
- OSWER (Office of Solid Waste and Emergency Response). (2007) Estimation of relative bioavailability of lead in soil and soil-like materials using in vivo and in vitro methods. OSWER 9285.7-77 ***(pH 1.5)***
- Ruby M. V., Schoof R., Brattin W., Goldade M., Post G., Harnois M., Mosby D. E., Casteel S. W., Berti W., Carpenter M., Edwards D., Cragin D. and W. Chappell. (1999). Advances in Evaluating the Oral Bioavailability of Inorganics in Soil for Use in Human Health Risk Assessment. Env. Sci. & Technol., 33, 21: 3697–3705 *(As based on EU Standard for Safety of Toys, Safety of Toys, Part 3: Migration of certain elements. 1994. European Standard EN 71-3, i.e.* ***pH 1.5)***
- Schroder J.L., Basta N.T., Casteel S. W., Evans T. J., Payton M. E. and J. Si (2004). Validation of the In Vitro Gastrointestinal (IVG) Method to Estimate Relative Bioavailable Lead in Contaminated Soils. J. Environ. Qual., 33: 513–521 **(*pH 1.8*)**
- Stopford W., Turner J., Cappellini D., Brock T. (2003). Bioaccessibility testing of cobalt compounds. J. Environ. Monit.,5: 675–680 ***(pH 1.5)***
- Walsh P.L., Bothe J.R., Bhardwaj S., Hu M., Nofsinger R., Xia B., Persak S., Pennington J. and Bak A. (2015). A canine biorelevant dissolution method for predicting in vivo performance of orally administered sustained release matrix tablets. Drug Dev Ind Pharm., 4:1-9. ***(pH 1-2)***

The selection of the gastric fluid composition in our current SOP (see Annexes) seems therefore justified and consistent with existing standards and available research data.

c) Sampling time

In our SOP extractions in gastric fluid were conducted for 2 h based on an average half time for gastric emptying of 17.7 min and complete emptying of 91 min in human volunteers (Tomlin et al., 1993; Wang et al., 2001). Sampling times of 1 to 2 hours have also been used in the following papers investigating bioaccessibility in surrogate gastric fluid.

- ASTM. (2007). Standard Test Method for Determining Extractability of Metals from Art Materials. ASTM. West Conshohocken.
- Brandon E.F.A., Oomen A.G., Rompelberg C.J.M, Versantvoort C.H.M., Van Engelen G.M. and A.J.A.M Sips. (2006). Consumer product in vitro digestion model: bioaccessibility of contaminants and its application in risk assessment. Regulatory Toxicology and Pharmacology, 44: 161-171.
- Brattin W., Drexler J., Lowney Y., Griffin S., Diamond G. and Woodbury L. (2013). An in vitro method for estimation of arsenic relative bioavailability in soil. Journal of Toxicology and Environmental Health, Part A , 76: 458-478.
- Denys S., Caboche J., Tack K., Rychen G., Wragg J., Cave M., Jondreville C. and C. Feidt (2012). In Vivo Validation of the Unified BARGE Method to Assess the Bioaccessibility of Arsenic, Antimony, Cadmium, and Lead in Soils. Environ. Sci. Technol., 46: 6252-6260
- Drexler J.W. and Brattin W.J. (2007). An in vitro procedure for estimation of lead relative bioavailability: with validation. Human and Ecological Risk Assessment, 13:383-401
- ESTCP (2012). Validation of an In Vitro Bioaccessibility Test Method for Estimation of Bioavailability of Arsenic from Soil and Sediment ***(Pg 12 Table 2-1: Overview of Published IVBA Procedures for Arsenic)***
- Hillwalker W.E. and Anderson K.A. (2014). Bioaccessibility of metals in alloys: Evaluation of three surrogate biofluids. Environmental Pollution, 185: 52-58. ***(Note: gastric tests were run on two residence times: 2h and 72h to calculate conservative risk rankings after incorporation in threshold ingestions. However, it is stated that use of gastric residence time longer than 3h is not physiologically appropriate – Twining J., McGlinn P., et al 2005. Risk ranking of bioaccessible metals from fly ash dissolved in simulated lung and gut fluids. Environ. Sci. Technol., 39: 7749-7756)***
- Molina R.M., Schaider L.A., Donaghey T.C., Shine J.P., Brain J.D. (2013). Mineralogy affects geoavailability, bioaccessibility and bioavailability of zinc. Environmental Pollution, 182: 217-224 ***(using the OSWER protocol for simulated gastric fluid extraction)***
- OSWER (2007). Estimation of Relative Bioavailability of Lead in Soil and Soil like materials using in vivo and in vitro methods **(*Pg on 70 Figure 3-2 Effect of temperature, time, and pH on IVBA (and pg 78 3.0 on information on gastric holding times in humans)***
- Schroder J.L., Basta N.T., Casteel S. W., Evans T. J., Payton M. E. and J. Si (2004). Validation of the In Vitro Gastrointestinal (IVG) Method to Estimate Relative Bioavailable Lead in Contaminated Soils. J. Environ. Qual. ,33: 513–521
- Rodriguez R.R., Basta N.T., Casteel S.W. and L.W. Pace (1999) An In Vitro Gastrointestinal Method To Estimate bioavailable Arsenic in Contaminated Soils and Solid Media. Environ. SciTechol., 33: 642-649
- Vasiluk L., Dutton M.D., Hale B. (2011). *In vitro* estimates of bioaccessible nickel in field-contaminated soils, and comparison with *in vivo* measurement of bioavailability and identification of mineralogy. Science of the Total Environment, 409: 2700-2706.

- Walsh P.L., Bothe J.R., Bhardwaj S., Hu M., Nofsinger R., Xia B., Persak S., Pennington J. and Bak A. (2015). A canine biorelevant dissolution method for predicting in vivo performance of orally administered sustained release matrix tablets. Drug Dev Ind Pharm., 4:1-9.

One-pager 4: Correlation *in vitro-in vivo*

Key to acceptance is the relationship bioaccessibility-bioavailability-toxicity. Do we have evidence linking bioaccessibility and bioavailability/internal exposure?

Publications on bioaccessibility *in vitro* tests have been searched and screened to extract the current state of the art of such tests in relation to *in vivo* bioassays.

Bioaccessibility is generally defined as the biologically relevant fraction of a chemical that is potentially available for uptake into a biological organism and the bioelution *in vitro* tests, have been used to account for the relative bioavailability of substances in several human health risk assessments (Henderson et al., 2012; U.S.EPA 2007, Brandon et al., 2006).

Human surrogate bio-fluids used in the bioelution test include gastro-intestinal, dermal, lung and internal implantation. Oral bioaccessibility is the most frequently investigated, both including a static gastric compartment (Drexler and Brattin, 2007; Stopford et al., 2003; U.S. EPA 2007) or dynamic gastro intestinal models (Garcia et al, 2001; Rodriguez and Basta, 1999; Ruby et al., 1996*).*

A preliminary literature search indicates that, the oral bioaccessibility model based on the static approach (gastric compartment as simple surrogate, pH 1.2-1.5, representing a worst case fasting exposure scenario, Hillwalker et al., 2014) has undergone extensive inter-laboratory robin testing (ASTM, 2007; Drexler and Brattin, 2007; EN 2002; U.S. EPA, 2009) as well as validation with *in vivo* studies of soil matrices. (Rodriguez and Basta, 1999; U.S. EPA, 2007).

In the following table, a concise summary of the articles and publications already screened is provided, reporting the conclusions on validation from the *in vivo-in vitro* investigations.

Overall, these tables show that for several metals (e.g. lead, arsenic, zinc, cadmium and nickel) there is good evidence **that bioaccessibility of metal ion in gastric fluid correlates with in vivo systemic bioavailability and/or toxicity**.

The literature search has identified additional publications and data presented in international forums like Asia Pacific Economic Cooperation (APEC), which support this conclusion, including for metals such as mercury and chromium (Lowney, APEC 2015).

	Type and settings materials	Correlation *in vitro-in vivo*	Reference
1	19 test materials were selected, mostly collected from residential soils, tailings, and slags from mining related waste sites across USA. Broad Pb concentration and diverse assemblage of relatively small (about 20µm) mineralogical forms of Pb (details provided) *In vitro* studies of Pb relative bioavailability (RBA) were investigated to identify alternative for estimating RBA of Pb in soil-like samples. Basis: rate/extent Pb solubilisation in gastrointestinal fluid is likely to be an important determinant of Pb bioavailability *in vivo* + most of *in vitro* tests are done in extraction solvent resembling gastric fluid. The fraction that solubilises is referred to as *in vitro* bioaccessibility. The Relative Bioaccessibility Leaching Procedure (RBALP) is reported as a *simple, reproducible and rapid in vitro* procedure for estimating *in vivo* (juvenile swine) relative bioavailability (RBA) of Pb in solid media.	*In vitro* measurements of bioaccessibility referred to in this publication correlate well with i*n* vivo measurement of RBA (gastro): linear regression correlation between *in vivo* Pb RBA estimates and *in vitro* Pb bioaccessibility estimates were established for 19 test materials. The results appear to be broadly applicable although further testing of a variety of different Pb forms is required to determine if exceptions exist. Drexler and Brattin reported that the performance of the method was evaluated by running triplicate analyses of each test substance, (using three independent laboratories). Results were then compared to RBA values as measured *in vivo.* The outcome is that RBALP measurements are strongly correlated with the *in vivo* RBA values (statistics provided too). Moreover, comparison of results within and between laboratories indicates that the procedure is highly reproducible with inter and intra-laboratory coefficients of variation of 4 and 6% respectively and within sample precision of approximately 7%.	OSWER. Office of Solid Waste and Emergency Response. (2007) Estimation of relative bioavailability of lead in soil and soil-like materials using in vivo and in vitro methods. 9285.7-77 (As presented by Drexler J.W., Brattin W.J., 2007). An in vitro procedure for estimation of lead relative bioavailability: with validation. Human and Ecological Risk Assessment, 13:383-401)

	Type and settings materials	Correlation *in vitro-in vivo*	Reference
2	Pb and As occur in soil as a complex mixture of solid phase chemical compounds of varying particle size and morphology. Spatial heterogeneity of such complex mixtures are reflected by variable metal bioavailability from soil at a site. In vivo data base for gastric testing are evaluated for Pb and As.	For Pb the correlation *in vitro-in vivo* is clearer than for As, primarily because As has a less comprehensive and reliable *in vivo* database The research indicates that the extent of Pb and As dissolution in the acidic environment of the stomach is predictive of relative bioavailability of these elements in animal models. NOTE: this study dates from 1999 and already recognises the validation of Pb bioaccessibility studies and the ongoing research on As bioavailability.	Ruby M.V. , Schoof R. ,Brattin W. ,Goldade M. , Post G. , Harnois M. , Mosby D. E., Casteel S. W., Berti W., Carpenter M., Edwards D., Cragin D.and W. Chappell (1999). Advances in Evaluating the Oral Bioavailability of Inorganics in Soil for Use in Human Health Risk Assessment. Env. Sci. & Technol., 33, 21: 3697–3705
3	The publication reports the results of a study performed to develop an *in vitro* bioaccessibility (IVBA) extraction technique for estimating the relative bioavailability (RBA) of As in soil. Several steps were implemented: (1) identification of up to 3 extraction fluid variables-having largest effect on measured As IVBA; (2) based on Step 1, test range of different extraction fluid (21) on 12 soils to see which will yield useful *in vitro-in vivo* correlations; (3) based on results from step 3, test selected 3 extraction fluids on large set of test soils (39) to select final extraction fluid leading to best IVIVC; (4) evaluate within and between lab precision of IVBA results using 12 different soils extracted with 2 different extraction fluids by 4 different labs.	For RBA values measured in swine, the best correlation (R2=0.72) was obtained using pH 1.5 extraction fluid (without phosphate or hydroxylamine additions). Protocols developed and tests details provided. The proposed model was also compared to other *in vitro* methods and identified advantages are listed and explained: e.g. the model is based on a larger data set of calibration samples (n=20 swine) to establish regression model; the use of diverse data set for IVIVC increasing confidence that correlation is likely to be applicable across a wide range of test materials; the model is based on extensive testing of extraction conditions; the interlab testing established within and between lab precision and showed high reproducibility; the simplicity of the method that uses single extraction steps & simple extraction fluid (in contrast with sequential extraction steps/creation of fluids that closely mimic complex GI fluids)	Brattin W., Drexler J., Lowney Y., Griffin S., Diamond G., Woodbury L. (2013). An in vitro method for estimation of arsenic relative bioavailability in soil. Journal of Toxicology and Environmental Health, Part A, 76: 458-478.

	Type and settings materials	Correlation *in vitro-in vivo*	Reference
4	A method was developed to simulate the human gastrointestinal environment and to estimate bioavailability of As in contaminated soil and solid media. 15 contaminated soils, collected from mining/smelter sites were analysed.	*In vitro* results were compared with *in vivo* RBA As. Arsenic extracted by *in vitro* gastrointestinal (IVG) method was not statistically different than relative bioavailable (RBA) arsenic measured by *in vivo* method.	Rodriguez R.R. and N.T Basta. (1999). An In Vitro Gastrointestinal Method To Estimate Bioavailable Arsenic in Contaminated Soils and Solid Media. Environ. Sci. Technol. ,33: 642-649
5	The study was conducted on 16 soils contaminated by smelting or mining activities, identified as soils with anthropogenic contamination, which are likely candidates for human health risk assessment. Specifically designed *in vivo* study with soils relevant to EU conditions along with better control on pH in the stomach phase leads to Unified BARGE Method (UBM), which produces bioaccessibility data that is a very good analogue of juvenile swine bioavailability measurements for As, Cd, Pb.	UBM test is able to assess the bioaccessibilities of As, Cd, and Pb in the contaminated soils that are studied. This study has addressed many of the issues arising from a preliminary interlaboratory trial of the UBM, but not yet addressed the *interlaboratory reproducibility* (problematic in Wragg et al, 2011) but authors recognise that further follow up on this will provide the *'last piece of evidence that the method can be used as a routine test in risk assessment studies*'.	Denys S., Caboche J., Tack K., Rychen G., Wragg J., Cave M., Jondreville C. and C. Feidt (2012). In Vivo Validation of the Unified BARGE Method to Assess the Bioaccessibility of Arsenic, Antimony, Cadmium, and Lead in Soils. Environ. Sci. Technol., 46: 6252-6260.

	Type and settings materials	Correlation *in vitro-in vivo*	Reference
6	The aim of the study was to develop, optimize, and validate an *in vitro* bioaccessibility (IVBA) method to estimate RBA of arsenic from soil for use in human health risk assessments. Arsenic IVBA is utilized to predict the *in vivo* RBA of arsenic in that sample. The method used in this study is reported as improved in "simplicity, reliability, and degree of validation compared to others". The used method is based on a single extraction step (no more to mimic different parts of gastrointestinal path)	Based on the regression model for swine data (reference: Phase IV/V report, Appendix B), it is expected that an RBA value estimated from IVBA is likely to be accurate within about 10% of the value that would have been obtained by measurement *in vivo*. *In vitro* and *In vivo* uncertainty: The study explains and compares limitations of *in vitro* and *in vivo studies*: the principal limitation of the *in vitro* method is that the RBA value predicted from an IVBA measurement may not be identical to the RBA value that would have been derived had an *in vivo* study been performed; however, *in vivo* RBA values have measurement error, which introduces uncertainty in to the estimate of the RBA, and the prediction error from the IVBA approach is presented as about the same magnitude as the measurement error in a typical *in vivo* RBA estimate. Also, the small number of soil samples usually assessed using *in vivo* methods introduces additional uncertainty in site-wide characterization of RBA because this small number of samples cannot allow assessment of variability in RBA across the site.	ESTCP Environmental Security Technology Certification Program (2012). Validation of an In Vitro Bioaccessibility Test Method for Estimation of Bioavailability of Arsenic from Soil and Sediment. ESTCP Project ER-200916

	Type and settings materials	Correlation *in vitro-in vivo*	Reference
7	The study investigates the effect of the dosing vehicle (e.g. dough) on the ability of the *in vitro* gastrointestinal (IVG) method to predict Pb RBA-associated with Pb soil ingestion. 18 contaminated soils from 8 different hazardous waste sites were evaluated. Pb bioavailability was also checked vs the presence of Fe, Ca, Zn affecting it and As and Cd. Absorption of Pb in fasted test subjects is about 10 times higher than in fed test ones (Ca/phosphates).	The ability of IVG to predict bioavailable Pb vs *in vivo* pig dosing trials is promising. Additional studies comparing *in vitro* results with *in vivo* bioavailable Pb are envisaged (on more soils from a wide range of matrices).	Schroder J.L., Basta N.T. ,Casteel S.W., Evans T.J., Payton M.E. and J. Si (2004). Validation of the In Vitro Gastrointestinal (IVG) Method to Estimate Relative Bioavailable Lead in Contaminated Soils. J. Environ. Qual., 33: 513–521
8	Various samples of Zn containing minerals, and one sample of weathered mine waste collected at site that was a former Pb, Zn mine. In vitro gastric fluid extraction was conducted as based on USEPA protocol for Pb and OSWER, 2007.	The trends observed in *in vitro* extractions and geochemically based sequential extractions were consistent with the *in vivo* results, confirming that solid phase speciation and geochemical alteration of speciation during weathering can have significant impact on biological uptake of Zn. *In vitro* tests can predict relative bioavailability of micronutrient metals as Zn.	Molina R.M., Schaider L.A., Donaghey T.C., Shine J.P. and J.D. Brain (2013). Mineralogy affects geoavailability, bioaccessibility and bioavailability of zinc. Environmental Pollution, 182, 217-224
9	In a study from Bradham et al .(2011), As RBA was determined in 9 contaminated soils (residential and smelter slag sites). In this study, those soils were used to assess relationship between As RBA and bioaccessibility via *in vitro* assays commonly used, i.e. SBRC, IVG, PBET, DIN, BARGE UBM	Linear regression models were established for the investigated (gastric) methods. Results of *in vivo* determinations of As relative bioavailability (RBA) were compared with As *in vitro* bioaccessibility (IVBA) results no significant difference in slopes of the relationships were found when SBRC, IVG, PBET, DIN and UBM gastric phase data were used.	Juhasz A.L., Smith E., Nelson C., Thomas D.J. Bradham K. (2014). Variability Associated with As in Vivo–in Vitro Correlations When Using Different Bioaccessibility Methodologies. Environmental Science & Technology 48:11646-11653.

	Type and settings materials	Correlation *in vitro-in vivo*	Reference
10	13 As-contaminated soils were assessed for As RBA (based on *in vivo* swine model) and As bioaccessibility (based on *in vitro:* Solubility Bioaccessibility Research Consortium gastric phase extraction; SBRC-G). *In vivo* and *in vitro* data were used to assess the validity of the As RBA-bioaccessibility correlation previously described by Juhasz et al. (2009).	Validation of *in vitro* method: As *in vivo-in vitro* correlation did not show significant difference to Juhasz et al. (2009) data ($P > 0.05$) indicating that the relationship between As RBA and As bioaccessibility was consistent. Linear regression was derived and cross validation methodologies used to determine the performance of the linear regression model.	Juhasz A.L., Herde P., Herde C., Boland J, Smith E (2014) Validation of the Predictive Capabilities of the Sbrc-G in Vitro Assay for Estimating Arsenic Relative Bioavailability in Contaminated Soils. Environ. Sci. Technol., 48(21): 12962–12969
11	*In vitro* bioaccessibility (IVBA) assays estimate arsenic (As) relative bioavailability (RBA) in contaminated soils to improve accuracy in human exposure assessments. In the study, a robust linear model was developed to predict As RBA in mice using IVBA, and the predictive capability of the model was independently validated using 40 As-contaminated soils (31 used for initial model development and 9 used for independent model validation). Soils varied in soil type and contaminant source.	The *in vivo-in vitro* correlation and independent data validation are presented to support validation of the method. The model development resulted in the linear equation model: RBA = 0.65 × IVBA + 7.8 and an R(2) of 0.81 was extracted.	Bradham K.D., Nelson C., Juhasz A.L., Smith E., Scheckel K., Obenour D.R., Miller B.W., and D.J. Thomas . (2014) Independent data validation of an in vitro method for the prediction of the relative bioavailability of arsenic in contaminated soils. Environ. Sci. Technol., 49(10): 6312-6318
12	Bioelution studies in gastric (2h) and intestinal (24h) fluids (in parallel) were conducted with 12 different nickel compounds. The same samples were tested in rat acute oral ingestion studies.	A good correlation between oral LD50 and bioaccessibility of nickel in gastric fluid was found. Additional consideration of the bioaccessibility results in intestinal fluid did not significantly improve the regression compared to using results from gastric fluid alone, Similarly, bioaccessibility of various nickel compounds in gastric fluid correlated with in vivo bioavailability reported by Ishimatsu and coworkers in rat absorption studies	Henderson RG, Durando J, Oller AR, Merkel DJ, Marone PA, Bates HK, (2012). Acute Oral Toxicity of Nickel Compounds. Regul Toxicol and Pharmacol. 62, pages 425-432. Henderson RG, Cappellini D, Seilkop SK, Bates HK, Oller AR, (2012). Oral Bioaccessibility Testing and Read-Across Hazard Assessment of Nickel Compounds. Regul Toxicol and Pharmacol. Volume 63, Issue 1, pages 20–28. Ishimatsu, S., Kawamoto, T., Matsuno, K., Kodama, Y., (1995). Distribution of various nickel compounds in rat organs after oral administration. Biol. Trace Elem. Res.49, 43–52.

One-pager 5: Sequential versus parallel testing

For oral fluids, possible consequences of testing metal substances independently in each oral fluid (e.g., saliva, gastric, intestinal) versus testing them sequentially? Under which circumstances would there be differences in releases when materials are tested in parallel settings (intestinal, gastric) or sequentially? Could gastric chloride interact with metal ions in a way that would affect the subsequent dissolution in intestinal fluid? We should however keep in mind the need to keep the test simple

A preliminary search of the literature was conducted to identify studies that looked at the sequential bioaccessibility of metals in saliva, and/or gastric, and/or intestinal phases compared to the bioaccessibility of metals in the gastric phase alone or the gastric and intestinal phases tested in parallel. The main conclusions from these studies are summarized briefly below.

- Oomen et al 2002: in this early summary report the authors stated*: "Apparently, **the gastric compartment is the crucial step in mobilizing the heavy metals from soil** although it is not the final step in the bioaccessibility process."*
- RIVM report 320102002/2004: "*Studies in animals and humans indicate that the bioavailability of compounds from food can be significantly different depending on the food source (food product), food processing or food preparation. As a consequence, a contaminant in product A can lead to toxicity whereas the same amount of contaminant in product B will not exert toxic effects*". This principle can easily apply to alloys. **Regarding the sequential test protocols examined and the metal bioaccessibility after shifting from gastric to intestine pH the report states: "*The increase in bioaccessibility of lead, cadmium and arsenic from Montana Soil was less [compared to B(A)p], 1.6- to 1.2-fold increase, respectively*."**
- Juhasz et al. 2011: "*In this study, As-contaminated soils (n=12) were assessed for As bioaccessibility using the Unified Bioaccessibility Research Group of Europe in vitro method (UBM) incorporating gastric, saliva-gastric or saliva-gastric-intestinal phases. Arsenic bioaccessibility was compared to previous published As relative bioavailability data for these soils to determine the correlation between in vitro and in vivo data. Comparison of in vitro and in vivo data indicated that the correlation between As bioaccessibility (UBM) and As relative bioavailability (swine assay) was similar irrespective of the in vitro phase used for its determination. The UBM incorporating all phases (saliva-gastric-intestinal) provided the best in vivo-in vitro correlation (slope=1.08; R(2)=0.59), however there was no significant difference in the goodness of fit (R(2) ranged from 0.48 to 0.59) or the slope of the lines (0.93-1.08) for either variation of the UBM (P=0.9946). This indicates that **there was no improvement in the As relative bioavailability predictive capabilities when the UBM was extended from a single gastric phase to saliva-gastric or saliva-gastric-intestinal phases**."*
- Deshommes et al 2012: "*Juhasz et al. 2009 showed that the solubility of PbAc salt decreases greatly under simulated intestinal conditions (pH 4–7.5), reflecting the gradual increase in pH at the entrance to the intestine: from nearly 100% at pH 1.5, the IVBA decreases markedly between pH 4 and 6 to about 14.3±7.2% at pH 6–7. Overall, initial concentrations in the range of 1 to 10 mg Pb/L did not influence solubility in the pH 1.5-7.5 range; slight differences were noted for high dosages of 5 and 10 mg/L with lower solubility at pH 5.5, and small but significant increases in solubility at pH 7.5. The steep decrease in PbAc solubility between pH 4.0 and 6.0 corresponds to the gradual increase in pH in the duodenum and jejunum, where most of the absorption and transport of Pb cations and complexes is supposed to take place".*

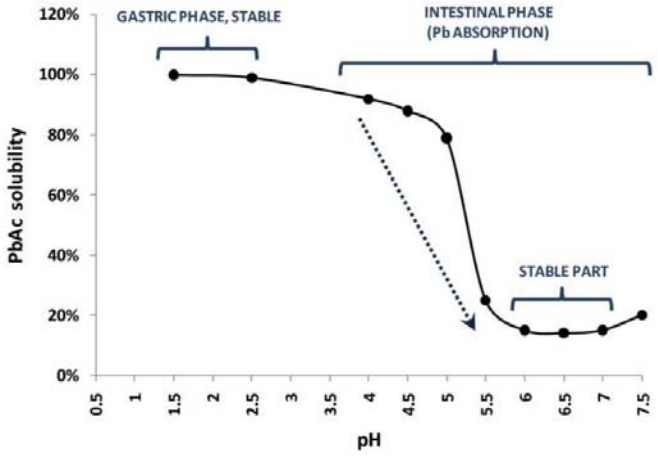

Figure 3. Changes in Pb acetate (PbAc, 1-10 mg Pb/L) solubility with pH, during the gastric and intestinal phases. Adapted from [67].

Therefore only very tiny additional solubilisation is to be expected when extending the test to mimic intestine conditions: "*A realistic representation of the intestinal phase would include gradually increasing the pH and performing a series of sample collections during this increase, but doing so would be a huge challenge and would introduce much variability.*"

- Denys et al 2012: *"For the cations in the "stomach" phase, the BAF (i.e. the stomach phase bioaccessible fraction) values were 99 ± 2% and 98 ± 3% for Pb-acetate and Cd-chloride, respectively. For the anions, the As BAF was 95 ± 3% and the Sb BAF was 93 ± 5%. This showed that all four elements were either indistinguishable or within 2% of being 100% bioaccessible for the reference compounds in this compartment. In contrast, in the "stomach and intestine" [combined] phase the cations had much reduced BAFs with Pb and Cd giving values of 66 ± 3% and 68 ± 3% with As and Sb BAFs of 92 ± 4% and 90 ± 2%, respectively. The lower recoveries of Pb and Cd can be explained by the fact that the behavior of these elements is strongly pH dependent. In the higher pH environment of the "stomach + intestine" phase these metals can precipitate from solution, be reabsorbed onto the soil, and complexed by pepsin. This is not observed in the case of elements (such as As and Sb) that form anions in solution and is consistent with previous studies*."

  Several references to the BARGE studies, within and outside Europe, were made at the BARC (Bioaccessibility Research Canada) workshop in 2011 : "***Gastric relative bioaccessibilities of Pb and Cd as measured by the UBM are accurate (and conservative) estimates of the relative bioavailabilities of these contaminants. For As, both gastric and gastro-intestinal bioaccessibility as measured by the UBM are accurate estimates of the relative bioavailability of this contaminant.*** "
- Li et al 2015. By modifying the assays, using SBRC, IVG, DIN, and PBET assays[11], the roles of different gastric components such as glycine, pepsin, mucin, phosphate, and citrate in different assays were elucidated. Variation in As bioaccessibility among assays was similar for house dust and soils, with SBRC assay providing the highest bioaccessibility in gastric phase. In intestinal phase, dissolved As was probably adsorbed onto precipitated iron oxides, causing a sharp decrease in As bioaccessibility by

[11] SBRC: Solubility/Bioavailability Research Consortium method; IVG: in vitro gastrointestinal method; PBET: physiologically based extraction test; DIN: Deutsches Institutfür Normunge.V. method; UBM: unified BARGE method.

SBRC assay Authors conclude that "***In general, the gastric phase of SBRC assay provided a more conservative assessment of As exposure than the intestinal phase of SBRC and other assays***."

- Laird et al 2013: To the knowledge of the authors, the 2007 study was the first study to demonstrate that intestinal microorganisms increase arsenic bioaccessibility in the human gastrointestinal tract. However in 2013 the same author concluded in another study: "*In vitro GI microbial activity in the distal small intestine increased arsenic release from soils; however, these effects were unlikely to be relevant since they were transient and demonstrated small effect sizes. In vivo arsenic absorption for juvenile swine was unaffected by antibiotic treatment.* ***Therefore, it appears that microbial effects on arsenic release do not result in increased arsenic bioavailability****. However, it remains to be seen whether the results for the limited set of soils described herein can be extrapolated to arsenic contaminated sites in general."*

Preliminary conclusion:

- Literature (mainly on metal solubilization from soils) shows that low pH yields the most conservative estimate of bioaccessibility. For some metals gastric release and sequential gastric-intestine release give comparable results as reported for the BARGE method. For Pb and Cd it is reported that these metals precipitate or build pepsin-complexes under intestine conditions and therefore a sequential testing is not recommended. Gastric biofluids from the static gastric compartment model are considered simple surrogates with low pH levels (pH 1.2-1.5) representing a worst-case fasting exposure scenario for a conservative bioaccessibility assessment (Hillwalker *et al.* 2014) and several models that have undergone "extensive inter-laboratory round robin testing and validation with *in vivo* studies with soil matrices" exist already (Rodriguez and Basta, 1999; U.S. EPA, 2007).
- A complete and realistic sequential testing would introduce many technical problems and a lot of variability and does not seem to add more conservatism to the approach. Brattin W., *et al.* (2013) developed and validated an *in vitro* method that utilizes a single extraction step as well as simple extraction fluids. By decreasing the complexity of the testing methodology and increasing the number and diversity of samples to identify optimal conditions for testing, run calibration and inter-laboratory testing to establish within & in between-lab precision, the authors developed and validated an *in vitro* test method to assess the relative bioavailability of metals from soils.
- Influence of microbes on the bioaccessibility of metals in the body is conflicting. There is no clear hint on the biological relevance of it.

To summarise: Gastric testing (alone) is recognized commonly as a conservative approach and should be used for bioelution testing from alloys to mimic the uptake of a given metal into the human body. Testing of gastric and intestinal (or saliva) fluid in parallel might be possible to make sure that metals that may have higher metal ion release at neutral than acidic pH are not missed.

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One-pager 6: Enforceability

Could we propose some ideas on how to achieve an easy, enforceable system: questions an inspector may ask, matrix of data and explanations he should be shown?

Please note that the proposal described in this one-pager covers only the use of bioaccessibility-related approaches for the classification of alloys human health endpoints /systemic effects that may occur after oral exposure. Inhalation- and local effects are not discussed.

Enforcement of alloy classifications should not be different than of other mixtures in that the enforcement authorities will expect a company to have documented procedures and an outline of the followed rationale for classification and labelling decisions.
This rationale should include references to any relevant ECHA guidance and documentation on the followed approach for classifying the mixture/alloy (see below).
A compliant eSDS should mention the classification method in case the CLP default mixtures approach is not followed, make reference to where documentation can be found and be accompanied by appropriate labels.

***

With regard to the documentation to be kept on-site and to be made available on request of inspectors, it is proposed to structure it as follows:

- **General information on the alloy:**
  - Short description of the composition of the alloy, its physical form
  - Short description of main property and expected technical function

- **Rationale followed to determine the classification** :
  - Approach followed:
    - ☐ CLP default approach based on content of hazardous component(s) in the alloy
    - ☐ Alloy-specific approach using existing test data on the alloy (not for the hazard classes germ cell mutagenicity', 'carcinogenicity' and 'reproductive toxicity')
    - ☐ Bioelution supported bridging approach
    - ☐ Bioaccessible concentration approach

  - If the **CLP default mixtures approach** has been used:
    - o List the constituents of the alloy with the associated classifications their reference (harmonized classification/self-classification)

Alloy name:	Constituent 1	Constituent 2	Constituent 3	Constituent 4	Constituent …
Constituent name					
Acute toxicity *Reference*					
Skin corrosion/irritation (incl. GCL/SCL) *Reference*					
Serious eye damage/irritation (incl. GCL/SCL) *Reference*					
Respiratory of skin sensitisation (incl. GCL/SCL) *Reference*					
Germ cell mutagenicity (incl. GCL/SCL) *Reference*					
Carcinogenicity (incl. GCL/SCL) *Reference*					
Reproductive toxicity (incl. GCL/SCL) *Reference*					
STOT-single exposure (incl. GCL/SCL) *Reference*					
STOT-repeated exposure (incl. GCL/SCL) *Reference*					
Aspiration hazard (incl. GCL/SCL)					
Hazardous to aquatic environment - ACUTE (incl. ERV/M-factors)					
Hazardous to aquatic environment - CHRONIC (incl. ERV/M-factors)					

- Explain how the classification has been determined for the alloy for every endpoint as well as the resulting H and P statements and labels

- **If alloy specific data has been used** (ONLY for endpoints which are not germ cell mutagenicity', 'carcinogenicity' and 'reproductive toxicity' hazard classes referred to in sections 3.5.3.1, 3.6.3.1 and 3.7.3.1 of Annex I of the CLP),
    - Explain evidence driving the classification

- **If the bioelution supported bridging approach** was used (extrapolation from properties from one source alloy to a target alloy), document:

    - identity of both source and target alloy and their respective compositions
    - classification of the source alloy: reference and approach used (if known), reliability?
    - justification for the choice of the source alloy (same hazardous constituents? Is there another information supporting the proposed grouping like standards, technical performance?)
    - how bioelution results were generated and used to bridge the classification:
        - keep the reports provided by the labs including a reference to the used protocol, medium, duration of exposure, sampling times, characteristics of test materials, …
        - were results for the target and source alloy generated simultaneously, in the same lab?
        - if not, were the test conditions for the target and source alloy comparable?
        - were the samples of the target and source alloy comparable (particle size, etc.)?
        - did the tests address all relevant constituents for classification? If not, is there a valid scientific justification?
        - how was the weight of evidence approach applied to bridge the classification from the source to the target alloy? Which data were considered in addition to bioelution results?

- If the **bioaccessible concentration approach** was used:

    - List the constituents of the alloy with the associated classifications their reference (harmonized classification/self-classification)

alloy name:	Constituent 1	Constituent 2	Constituent 3	Constituent 4	Constituent …
Constituent name					
Acute toxicity *Reference*					
Skin corrosion/irritation (incl. GCL/SCL) *Reference*					
Serious eye damage/irritation (incl. GCL/SCL) *Reference*					
Respiratory of skin sensitisation (incl. GCL/SCL) *Reference*					
Germ cell mutagenicity (incl. GCL/SCL) *Reference*					
Carcinogenicity (incl. GCL/SCL) *Reference*					
Reproductive toxicity (incl. GCL/SCL) *Reference*					
STOT-single exposure (incl. GCL/SCL) *Reference*					
STOT-repeated exposure (incl. GCL/SCL) *Reference*					
Aspiration hazard (incl. GCL/SCL)					
Hazardous to aquatic environment - ACUTE (incl. ERV/M-factors)					
Hazardous to aquatic environment - CHRONIC (incl. ERV/M-factors)					

Note: this table includes all relevant constituent(s) classifications for the mixtures classifications. Endpoints where the Bioaccessible Concentration approach -as currently discussed- might apply are highlighted (systemic effects, oral exposure)

Document:

- how oral bioelution results were generated and used:
  - keep the reports provided by the labs including a reference to the used protocol, medium, duration of exposure, sampling times, characteristics of test materials, …
  - check the identity of and justification for the selection of reference substance
  - were the results for the reference substance and the alloy generated in the same lab?
  - if not, were test conditions for the reference substance and the alloy comparable?
  - were the samples of the reference substance and the alloy comparable (particle size, etc.)?
  - document how the calculation of the bioaccessible concentration of the hazardous components in the alloy has been done
- how was the weight of evidence approach applied to include the oral bioelution results in the derivation of the classification?
- does the bioelution test address all relevant constituents, if not is there a valid scientific justification?

Note: if the MeClas (www. Meclas.eu) tool has been used, keep at hand the MeClas report and intermediate calculations