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Oral bioaccessibility testing and read-across hazard assessment of nickel compounds

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ABSTRACT

In vitro metal ion bioaccessibility, as a measure of bioavailability, can be used to read-across toxicity information from data-rich, source substances to data-poor, target substances. To meet the data requirements for oral systemic toxicity endpoints under the REACH Regulation in Europe, 12 nickel substances underwent bioaccessibility testing in stomach and intestinal fluids. A read-across paradigm was developed based on the correlation between gastric bioaccessibility and *in vivo* acute oral toxicity. The oral LD_{50} values were well predicted by nickel release ($R^2 = 0.91$). Samples releasing <48% available nickel (mg Ni released/mg available Ni × 100) are predicted to have an $LD_{50} > 2000$ mg/kg; while samples releasing >76% available nickel are expected to have an LD_{50} between 300 and 2000 mg/kg. The hazard classifications (European Regulation on Classification, Labelling and Packaging of Chemical Substances and Mixtures) for all oral systemic endpoints were evaluated based on read-across from three source nickel compounds (sulfate, subsulfide, oxide). Samples releasing <48% available nickel were read-across from nickel oxides and subsulfide. Samples releasing >76% Ni were read-across from nickel sulfate. This assessment suggests that nickel chloride and dihydroxide should be less stringently classified and nickel sulfamate should receive a more stringent classification for oral systemic endpoints than currently assigned.

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1. Introduction

Read-across can be defined as, "the use of hazard specific information for one substance ("source") to predict the same hazard for another substance ("target"), which is considered to have similar physical–chemical environmental fate and/or (eco)toxicological properties" (EC, 2009b). Various applications of read-across have become particularly useful for filling data gaps and meeting new regulatory demands such as the Registration, Evaluation, and Authorization of Chemicals (REACH) Regulation in Europe (EU) (EC, 2006). For the nickel substances included in the present study, the submission requirements included a substantial data-set ranging from acute toxicity and irritation studies to repeated dose and reproductive toxicology studies (EC, 2009b; ECHA, 2008). Therefore, an extensive data extrapolation or "read-across" program was developed for these pure nickel substances under REACH to fulfill higher-tier data requirements for various routes of exposure and health endpoints. In the past, water solubility has been used as a basis for grouping metallic compounds including nickel compounds for the purposes of read-across for classification of all health endpoints. For some nickel compounds, water solubility-based classifications without confirmatory *in vivo* data were included in the first ATP to the European Regulation on Classification, Labelling and Packaging of Chemical Substances and Mixtures (CLP; EC, 2009a,b). However, any scientifically robust read-across paradigm must take into account the bioavailability of the nickel ion at target sites relevant to each route of exposure. The read-across approach presented here for the oral systemic toxicity of pure nickel substances is consistent with the approaches identified by the REACH Regulation and complies with one of its main drivers: a reduction in the extent of animal testing utilized in performing hazard and risk assessments (EC, 2006; ECHA, 2008).

The ability to perform scientifically valid read-across of data from one well characterized, data-rich, source substance to a target substance with little or no data requires that a minimum amount of information pertaining to the target substance be compared to the same information from the source substance (EC, 2009b). In the case of metal-containing substances, it is often the case that the metal ion is the responsible entity for the observed toxicity of the compounds. As the simple presence of a metal in a substance does not necessarily impart to that substance the biological

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properties of the metal ion, comparison of bioavailability data can be utilized to perform read-across assessments for metal substances. Bioavailability, defined as the fraction of the dose that reaches systemic circulation, is key for determining toxicity (Klaassen, 2001). Ideally, comprehensive information on the bioavailability of metal substances is derived from *in vivo* toxicokinetic or toxicological tests. When the bioavailability of a metal substance is not known, the amount of ion available for absorption may be estimated using *in vitro* methods. One such method involves the determination of released metal ions in synthetic fluids. This *in vitro* dissolution is termed "bioaccessibility" and can provide a surrogate measure of the amount of a substance (e.g., metal ion) available for absorption (Ruby et al., 1999).

For metals, comparison of *in vitro* metal ion bioaccessibility data on source and target substances can be utilized to perform read-across assessments when it can reasonably be assumed that the "counter-ion" does not contribute to the observed toxicity. Analyses and read-across should be conducted for each toxicity endpoint (as opposed to reading-across all endpoints from one single source substance) and should be considered for each route of exposure since the chemical conditions of the gastro-intestinal tract, respiratory tracts and dermal systems are unique and can affect different chemicals in different ways. Bioaccessibility data are therefore used as the basis for identifying the most appropriate source compound for reading across each toxicity endpoint to a target substance or category of substances. Other relevant information, when available, should be included in the overall assessment of metal substances including physico-chemical properties, particle size and structure, temperature of formation, and toxicokinetics. As with any chemical category grouping and read-across method, the *in vitro* bioaccessibility results should be verified by comparison with *in vivo* bioavailability or toxicity studies, using the same or similar test material (OECD, 2007).

As discussed below, bioaccessibility-based read-across has already been employed for several metals in different matrices, where the chemical form of the metal has an impact on the toxicological properties of the substances being tested. These programs enable hazard and risk assessments of data-poor substances while substantially decreasing the time, costs, and number of animals normally required for *in vivo* bioavailability and toxicity studies. Despite small differences in methodologies, these studies all rely on some measure of bioaccessibility to estimate the bioavailability of metals and perform hazard and/or risk assessment.

One of the most extensively studied examples is the bioaccessibility of metals present in soils. Drexler and Brattin (2007) have published results of a round-robin validation of a relative bioaccessibility leaching procedure (RBALP), based on work generated by the U.S. EPA (2006), for estimating *in vivo* bioavailability of lead from soils. This physiologically-based extraction technique (PBET) utilizes an experimental system that incorporates parameters representative of the human gastrointestinal tract such as pH, mixing, and emptying rates. Data from this study together with *in vivo* verification testing in juvenile swine has been published (Ruby et al., 1996; Casteel et al., 2006). The Bioaccessibility Research Group of Europe (BARGE) recently published the outcome of an inter-laboratory study on the Unified BARGE Method (UBM) for lead, cadmium, and arsenic (Wragg et al., 2011). This method looks at the bioaccessibility of metals from soils in synthetic saliva, gastric and intestinal fluids. Other studies have investigated the bioaccessibility of metals from soil (Ellickson et al., 2001), household dust (reviewed in Turner, 2011), welding fumes (Berlinger et al., 2008), mine waste (Gray et al., 2010) and fly ash (Twining et al., 2005).

With regards to studies specifically evaluating the bioaccessibility of nickel, an extensive data set has been published on the bioaccessibility of nickel and other metals from stainless steels and other alloys using various forms of synthetic media (Hedberg et al., 2010; Herting et al., 2008; Midander et al., 2010). These studies have highlighted the influence of surface area and surface characteristics on the metal ion release from elemental forms of metals (e.g., alloys). Besides the characteristics of the sample itself, the bioelution protocol (e.g., loading, pH, agitation) may influence the rates of metal release and therefore, it is important to consider both sample and protocol information before comparing results from different studies. Guidance on how to use bioaccessibility data to perform hazard and risk assessments for alloys is currently under development (Health Risk Assessment Guidance for Metals (HERAG) Alloys Fact Sheet; http://www.icmm.com). The bioaccessibility of nickel from soils using synthetic gastric juice was investigated by Hamel et al. (1998). More recently, the oral bioaccessibility of nickel from field-contaminated soils in a PBET system and cultured intestinal epithelial cells was compared to bioavailability data for nickel from rat studies (Vasiluk et al., 2011).

We have previously published data on the release of nickel from nickel-containing substances in synthetic lung fluids (Oller et al., 2009) and have also conducted additional studies investigating the bioaccessibility of various nickel substances in other synthetic bodily fluids such as a lysosomal fluid and sweat (unpublished data). The bioaccessibility assays utilized in the present study were based on work carried out on cobalt substances as part of the risk assessment of consumer products (Stopford et al., 2003; Brock and Stopford, 2003). These methods are currently undergoing inter-laboratory validation using six different metals and metal-containing substances (including nickel sulfate hexahydrate and a nickel-containing alloy) in a total of five laboratories located in Belgium, Chile, Sweden, and the USA.

We report here the outcome of bioaccessibility testing of nickel substances in stomach and intestinal fluids, and the development of a read-across paradigm for oral systemic toxicity based on the correlation between bioaccessibility data and *in vivo* acute oral toxicity data (Henderson et al., 2012). The application of this analysis to the read-across assessment of data-poor nickel substances and its potential impact on hazard classification in the EU are also discussed. Three nickel compounds were used as source substances: nickel sulfate, nickel subsulfide and nickel monoxide (high calcining temperature). For these three compounds, an extensive database, including inhalation and/or oral studies looking at repeated toxicity, carcinogenicity, mutagenicity, and reproductive toxicity endpoints, is available. The target compounds included nickel acetate, nickel chloride, nickel dihydroxide, nickel fluoride, nickel hydroxycarbonate, nickel sulfamate and nickel sulfide. For some of the target compounds, acute toxicity studies were available, but studies of repeated toxicity were lacking for the majority of these compounds.

2. Materials and methods

2.1. Materials

Twelve different samples representing eleven distinct nickel compounds were tested. Test substances with their respective chemical formulas, purity and CAS numbers are listed in Table 1. All nickel substances were provided by members of the Nickel REACH Consortia (www.nickelconsortia.org) except for nickel sulfate hexahydrate (Sigma–Aldrich; St. Louis, MO), nickel sulfide (Alfa Aesar; Ward Hill, MA). Nickel content was determined by PIXE (Particle Induced X-ray Emission) or ICP-MS (Inductively Coupled Plasma Mass Spectroscopy). The particle size distribution was either measured using a Malvern Mastersizer 2000 or was reported by the supplier. Mean particle size was below 100 μm mean particle diameter for all samples except for the nickel oxide sample N112 (Table 1).

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Table 1
Description of the nickel substances used in this study: CAS number, Ni content, purity, particle size ($d_{0.5}$) and surface area.

Test substance	Sample ID	CAS No	Ni content (%)[a]	Purity (%)[b]	$d_{0.5}$ (μm)[c]	SA (m^2/g)[d]
Ni acetate tetrahydrate	N103	6018-89-9	24	100	NR	NR
Ni chloride hexahydrate	N98	7791-20-0	25	>97	NR	NR
Ni dihydroxide	N106	12054-48-7	54	>99	11.36[f]	9.66[f]
Ni fluoride tetrahydrate	N111	13940-83-5	32	99	NR	NR
Ni hydroxycarbonate	N109	12122-15-5	49	100	7.47[f]	391.5[f]
Ni oxide (green)	N9[e]	1313-99-1	77	NR	2.64	2.62
Ni oxide (green)	N46[e]	1313-99-1	62[g]	NR	NR	0.16[g]
Ni oxide (green)	N112	1313-99-1	81	98	467.9	0.01
Ni oxide (black)	N105	1313-99-1	75	96.4	15.6	96.09
Ni subsulfide	N18	12035-72-2	70	NR	1.68	2.55
Ni sulfamate tetrahydrate	N104	13770-89-3	18	100	NR	NR
Ni sulfate hexahydrate	N58	10101-97-0	23	⩾99	NR	NR
Ni sulfide	N97	16812-54-7	59	100	7.23	0.71

NR, not reported
[a] Ni content determined by PIXE or ICP-MS.
[b] Composition information as provided by supplier.
[c] Particle size measured with Malvern Mastersizer laser diffractor by Particle Technology Labs, Ltd., USA, unless otherwise noted.$d_{0.5}$ corresponds to the median particle diameter from the volume (mass) distribution.
[d] Surface area measured by B.E.T. gas absorption methodology. Measurements conducted at Particle Technology Labs, Ltd., USA, unless otherwise noted.
[e] N9 and N46 are archival related samples, sample N9 was used in bioelution studies, while sample N46 was used in *in vivo* studies (Henderson et al., 2012). The more recently acquired sample N112 is considered to be the representative sample for green NiO under REACH.
[f] Particle size and SA information provided by the supplier.
[g] Ni content and SA information gathered at Vale laboratories (Canada).

2.2. Bioaccessibility assays

The composition of the synthetic gastric (pH 1.5) and intestinal (pH 7.4) fluids and their respective bioelution protocols follow closely those of Stopford et al. (2003), with some modifications as described in Table 2. Briefly, extractions were performed for 2, 5, 24, and 72 h in a 37 °C shaker water bath using 0.1 g of sample in 50 ml of fluid. Filtered extracts were analyzed for released nickel using ICP/MS. The limit of quantitation (LOQ) was 25 μg Ni/g sample for both fluids.

The loading used here is 10-fold lower than the initial loading described in Stopford et al. (2003). Stopford and colleagues took a stepwise approach in which they started with a higher loading (1 g/50 ml, 20 g/l), corresponding to the initial loading recommended by ASTM D5517 (ASTM, 2003) and then utilized a lower loading of 0.1 g/50 ml if the initial loading resulted in greater than 5 ppm of metal released into solution. This was done to avoid erroneous low values caused by mass ion effect. Again this is consistent with the ASTM D5517 which states: "If results are greater than 250 ppm, the extractant volume should be increased to 100 ml or greater to keep metal levels in the eluate at a level of 5 ppm or less." The influence of loading on Ni release in gastric fluid was studied by Turner (2011). Increased fraction of Ni released at loadings lower than 1 g/l (<2-fold difference in release) were reported, even though the range of loadings relevant for oral exposure was listed as 2–10 g/l (Turner, 2011). In this study, an intermediate loading of 2 g/l was found to be a reasonable compromise to minimize mass ion effects and agglomeration while at the

Table 2
Description of the bioleution fluids and protocols.

	Bioelution gastric fluid	Bioelution intestinal fluid
Composition of fluid	0.07 N hydrochloric acid (HCl)	6.79 g/l sodium chloride (NaCl) 0.52 g/l potassium chloride (KCl) 1.85 g/l sodium bicarbonate ($NaHCO_3$) 0.11 g/l calcium chloride ($CaCl_2$) 1.07 g/l sodium taurocholate
Fluid pH	⩽1.5	7.4 ± 0.2
Bioaccessibility protocol	Each extraction was performed using 0.1 g of sample in 50 ml of bioleution gastric fluid. Samples were weighed into acid washed 250 ml amber Erlenmeyer flasks[a]. Gastric fluid was added to the flasks and they were then swirled to mix compound and fluid. The pH was checked and adjusted if necessary with 2 N HCl. The openings of the flasks were covered with parafilm and aluminum foil. The flasks were then placed in a preheated 37 °C reciprocal shaker bath (stroke length = 1"; 150 oscillations/min). The samples were allowed to shake for the required extraction times. Once complete, the flasks were removed from the bath. The pH was checked and the entire volume of the solutions was filtered through a 0.45 μm filter. The filtrates were collected in 8 oz. disposable plastic bottles and kept in a 35 °C incubator until they were analyzed.	Each extraction was performed using 0.1 g of sample in 50 ml of bioleution intestinal fluid in an acid washed 250 ml amber Erlenmeyer flask[a]. The pH of the solution was checked and adjusted if necessary with 2 N HCl or 1 N NaOH. To keep the extraction pH at 7.4, 5% CO_2 in nitrogen was bubbled into the solution at a rate of 50 cc/min. The bubbling solutions were placed in a preheated 37 °C reciprocal shaker bath (stroke length = 1"; 150 oscillations/min). The flasks were bubbled and allowed to shake for the required extraction time. Once complete, the flasks were removed from the bath. The entire volume of the solutions was filtered through a 0.45 μm filter and the pH was verified. The filtrates were collected in 8 oz. disposable plastic bottles and kept in a 35 °C incubator until they were analyzed.
Nickel analysis (LOD)	EPA Method #200.8 (ICP/MS) (0.05 mgNi/l)	EPA Method #200.8 (ICP/MS) (0.05 mgNi/l)

LOD: limit of detection.
[a] Glassware was soaked in 20% nitric acid overnight, followed by overnight soaking in deionized (DI) water. Finally glassware was rinsed with fresh DI water and oven dried.

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Table 3
Bioaccessibility: release of Ni ion in synthetic bodily fluids after 2, 5, 24, or 72 h (% of available Ni content).

Test substance	Sample ID	Dissolution in gastric fluid (%)[a]				Dissolution in intestinal fluid (%)[a]			
		2	5	24	72	2	5	24	72
Ni sulfate hexahydrate	N58	90.55	100.50	NC	NC	42.00	46.00	58.00	57.10
Ni chloride hexahydrate	N98	89.85	90.25	90.94	92.15	28.45	33.75	38.45	33.45
Ni acetate tetrahydrate	N103	88.50	91.50	92.70	93.75	28.85	31.10	38.50	39.60
Ni hydroxycarbonate	N109	84.30	85.50	85.70	86.00	0.76	0.76	1.50	1.35
Ni sulfamate tetrahydrate	N104	83.40	84.00	84.25	84.00	4.20	9.05	11.75	29.05
Ni fluoride tetrahydrate	N111	82.35	83.15	82.80	86.40	62.60	77.40	74.05	78.35
Ni oxide (black)	N105	29.60	78.25	80.50	81.70	0.11	0.13	0.32	0.37
Ni hydroxide	N106	26.30	60.35	78.90	85.70	0.01	0.01	0.01	0.02
Ni subsulfide	N18	22.65	28.90	33.30	49.15	0.08	0.19	0.25	0.26
Ni sulfide	N97	9.75	13.55	22.70	38.10	0.02	0.06	0.18	0.33
Ni oxide (green)	N9	0.33	0.52	1.05	2.00	0.08	0.09	0.12	0.12
Ni oxide (green)	N112	<LOQ[b]	0.00	0.03	0.03	0.01	0.01	0.01	0.01

NC, not conducted. Extractions were stopped after 100% dissolution observed.
[a] Reported values are mean values from duplicate experiments. Blank fluids were extracted in duplicate through the entire process usually at the shortest time period. Blank results were always below the LOQ. Variability among duplicate samples was less than 6%.
[b] LOQ, limit of quantitation equal to 25.0 μg Ni/g sample. Substances releasing Ni ion below the LOQ are considered to have 0.00% bioaccessibility.

Table 4
Summary of data incorporated into read-across verification and assessment: gastric bioaccessibility and oral acute toxicity.

Test substance	Sample ID	Gastric bioaccessibility- 2 h (μg Ni/g sample)[a]	Gastric bioaccessibility- 2 h (% Ni content released)[b]	Acute toxicity (oral LD_{50}; mg/kg bw)[c]
Ni sulfate hexahydrate[d]	N58	201,670	90.55	362
Ni chloride hexahydrate	N98	215,632	89.85	500
Ni acetate tetrahydrate	N103	209,810	88.50	550
Ni hydroxycarbonate	N109	408,690	84.30	2000
Ni sulfamate tetrahydrate	N104	147,783	83.40	1098
Ni fluoride tetrahydrate	N111	264,930	82.35	310
Ni oxide (black)	N105	222,328	29.60	9990
Ni hydroxide	N106	142,693	26.30	5000
Ni subsulfide[d]	N18	158,185	22.65	>11000
Ni sulfide	N97	57,485	9.75	NC
Ni oxide (green)[d]	N9, N46[e]	2540	0.33	>11000
Ni oxide (green)[d]	N112	<25	<LOQ[f]	>11000

NC, not completed.
[a] Bioaccessibility reported as μg Ni released per gram of sample. Reported values are mean values from duplicate experiments.
[b] Bioaccessibility reported as the percent of available Ni content released in synthetic gastric fluid after 2 h. Reported values are mean values from duplicate experiments. Different shading indicates a possible pre-grouping of the samples based solely on % Ni content released in gastric fluid after 2 h.
[c] Henderson et al. (2012).
[d] Source substance for purposes of read-across.
[e] The N9 sample was used in bioleution studies and the N46 sample in the *in vivo* studies.
[f] LOQ, limit of quantitation equal to 25.0 μg Ni/g sample. Substances releasing Ni ion below the LOQ are considered to have 0.00% bioaccessibility.

same time being relevant for the acute oral dosing in animals (approximately 0.1–5 g/animal).

2.3. Acute animal toxicity studies

In vivo acute oral toxicity studies conducted with all of the compounds included here except nickel sulfide are reported in a companion manuscript (Henderson et al., 2012). Summary data on LD_{50} values are provided in Table 4.

2.4. Statistics

Least squares regression analysis was used to investigate the relationship between nickel release and oral LD_{50} values. Alternative power transformations (e.g., LD_{50}^{x}) were used to evaluate nonlinearity in the relationship and to stabilize variances across the nickel release prediction range. The percentage of variation explained by the regression (R^2) was used to assess the goodness of fit. One-sided 95% upper and lower confidence limits for individual predicted LD_{50} values were constructed using standard least squares regression methodology. Statistical calculations were performed using SAS® software, Version 9.2.

3. Results and discussion

3.1. General approach

For metal compounds, comparison of *in vitro* bioaccessibility data as a surrogate for *in vivo* toxicokinetic or toxicological data on source (data-rich) and target (data-poor) substances can be utilized to perform read-across assessments. This is valid when it can reasonably be assumed that the toxic moiety is the metal ion and the counter-anion does not contribute to the observed toxicity. For systemic effects it is assumed that it is the absorbed dose of metal ion that will be related to the known toxicity of source metal compounds. This is the case for nickel (Ishimatsu et al., 1995; Henderson et al., 2012) and it has also been demonstrated to be the case for other metals (e.g., Lauwerys et al., 1994; Mushak, 1993). These source compounds can provide a reference from which to extrapolate their existing toxicological data to target

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substances based upon relative bioaccessibility. The bioaccessibility-based read-across approach reported here incorporates the following steps:

Step 1: Generate soluble nickel species release data in the appropriate fluids related to the route of exposure in question (in this case, oral) for source and target substances. At this stage, a preliminary grouping can be done based on these data.
Step 2: Incorporate *in vivo* toxicity or toxicokinetic data in order to verify that the bioaccessibility data correlate with toxicity and to generate a reference range.
Step 3: Assess the most appropriate grouping of substances and identify the source substance for each category or target substance based on relative bioaccessibility data.
Step 4: Use the new paradigm to read-across toxicological data from source substances to target substances based on relative bioaccessibility.

Bioaccessibility data via the oral route of exposure can provide important information regarding the potential bioavailability and subsequent toxicity for oral systemic endpoints including acute and chronic oral toxicity and reproductive toxicity. We have combined bioaccessibility data in synthetic gastric fluid with acute oral toxicity data to develop a read-across standard curve to enable the extrapolation of the systemic toxicities of source nickel compounds (nickel sulfate, nickel oxide, and nickel subsulfide) to target nickel compounds. This paradigm was utilized to meet data requirements under the REACH Regulation in Europe.

3.2. Bioaccessibility data (step 1)

To investigate bioavailability via the oral route of exposure, source and target nickel substances were subjected to bioaccessibility testing in two relevant synthetic biological fluids, gastric and intestinal. Data are reported in Table 3 as the percent of total available nickel ion released for extractions performed at all four time points.

Gastric release data after 2 h expressed as μg Ni/g sample are listed in Table 4 together with percent of available nickel released. Based on the latter data, presented in order of increasing gastric nickel (II) bioaccessibility, a pre-grouping of substances can already be done (see differential shading in Table 4). The first six substances include those considered to be water-soluble (nickel sulfate, nickel chloride, nickel acetate, nickel sulfamate, and nickel fluoride) and nickel hydroxycarbonate, all of which demonstrated nearly complete dissolution in gastric fluid within the first 2 h. For the water soluble substances, only a slight increase in nickel ion release was observed by 72 h ($\leqslant$10%) compared to the release after 2 h. The amount of nickel ion released from the water soluble substances in intestinal fluid varied, with most samples ranging from 29% to 78% of nickel content released after 72 h. Conversely, the nickel hydroxycarbonate sample released only 1.35% of available nickel content in intestinal fluid which is consistent with the testing results of this sample in other solutions with a neutral pH such as synthetic sweat (unpublished data). Similarly, we found a release of 0.7% of available nickel after extraction for 24 h in synthetic alveolar fluid for a sample of nickel carbonate (Oller et al., 2009). Although little variation among duplicate samples in synthetic intestinal fluid was found under the conditions of our assay, it is recognized that the results in intestinal fluid may be affected by small changes in pH or on the loading rates used. Therefore, although the intestinal fluid results presented here are considered representative of relative nickel release, further studies would be recommended if the intestinal bioleution data were to be used as the basis for a read-across program.

The remaining test substances include oxidic and sulfidic nickel compounds, which are considered to be water-insoluble or sparingly soluble, respectively. The gastric nickel (II) bioaccessibility from these substances at the earliest time point did not exceed 30% of available nickel, with the most refractory of these materials, green NiO, releasing essentially 0% of available nickel. While the results for green NiO did not change over time, an increase in nickel ion release was observed over time for the other substances. No appreciable release was measured from these substances in the neutral intestinal fluid (all <1%). The higher Ni release in gastric fluid compared to intestinal fluid cannot be explained based on the presence of complexing or chelating agents (e.g., citric acid), or the precipitation at neutral pH, and therefore the most likely explanation for the higher dissolution in gastric fluid is the difference in H^+ concentration. The effect of pH on the release of nickel depending on chemical form of the substance is consistent with other recently published data (Oller et al., 2009; Vasiluk et al., 2011).

In general, nickel release in synthetic intestinal fluid was much lower than that in synthetic gastric juice, even for the water-soluble substances. For each substance where nickel ion release in the intestinal fluid was >1%, the substance had already released 82–91% of its available nickel ion after the first 2 h in gastric fluid. In this case, of the remaining small percentage of nickel ion still available for release after passing through the stomach, only a small percentage would be further released and available for absorption in the intestines. Regardless of the fluids utilized, bioaccessibility methods do not take into account absorption, hence they are generally considered to overestimate bioavailability and toxicity.

3.3. Relationship between gastric bioaccessibility and oral toxicity data (step 2)

The question of what compartment(s) (e.g., stomach, intestine) to include as a basis for performing read-across has been evaluated by others, particularly with respect to lead and cadmium bioavailability in soils. Some studies have relied solely on assessment of the bioaccessible fraction in gastric fluid (Drexler and Brattin, 2007; reviewed in Turner, 2011). Several of the more extensive models used for assessing oral bioaccessibility incorporate additional compartments such as saliva and intestinal fluids, digestive enzymes, organic acids, bile salts, or cell culture (Oomen et al., 2003; Ellickson et al., 2001; Wragg et al., 2011). The conclusions made in these studies varied depending upon the strength, or lack thereof, of the correlation found between the *in vitro* dissolution data and *in vivo* toxicity or toxicokinetic data. Because of the different physicochemical properties of the various metal ions (e.g., acidity/alkalinity, complex formation abilities, etc.) it is clear that no generalizations can be made and the selection of the most appropriate fluid to use for bioaccessibility-based grouping and read-across has to be independently established for each metal.

In order to verify what bioaccessibility data provided the best estimate of systemic bioavailability of nickel and hence toxicity, acute oral toxicity studies in rats were carried out for all compounds tested here except for nickel sulfide (Henderson et al., 2012). A summary of the LD_{50} values from these studies is reported in Table 4.

For purposes of conducting the read-across assessment, correlations between acute toxicity data expressed in terms of LD_{50} (mg substance/kg bw) and nickel ion release (as percent of available nickel) in gastric and intestinal fluids and for different lengths of exposure were examined (all data not shown). We also examined the correlation between acute toxicity and the calculated total nickel release after 2 h in gastric fluid and subsequent 24 h in intestinal fluid. Nickel ion release after 2 h in synthetic gastric fluid resulted in a reasonable correlation ($R = -0.95$, $R^2 = 0.91$, Fig. 1),

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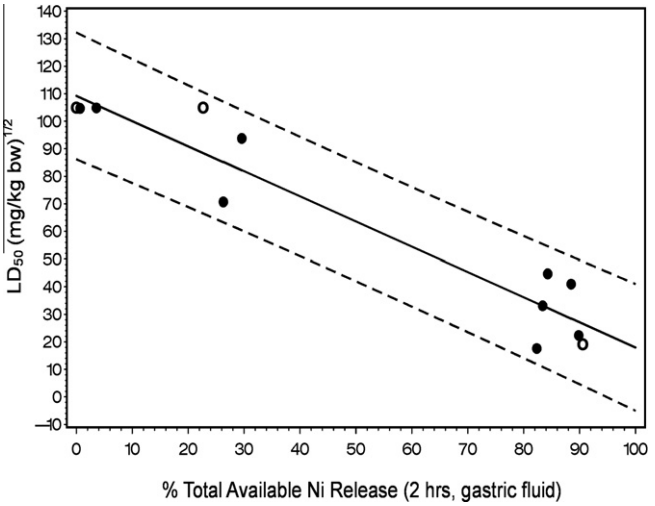


Fig. 1. Correlation between gastric bioaccessibility (% Ni content released, 2 h) and acute oral toxicity ($LD_{50}^{1/2}$). Solid line = regression fit; dashed lines = upper and lower one sided 95% confidence limits for individual sample values; open circles = source Ni substances, black circles = target Ni substances. $R^2 = 0.91$.

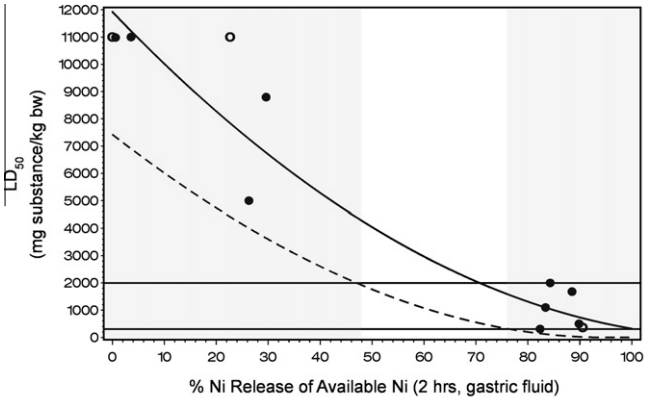


Fig. 2. Relationship between LD_{50} and Ni release, with associated lower confidence limits for individual predicted LD_{50} values. Solid line = regression fit; dashed line = one sided lower 95% confidence limits for individual sample values; open circles = source Ni substances, black circles = target Ni substances. Horizontal lines correspond to concentration cut-off limits for classification according the CLP system (300 and 2000 mg/kg bw). Shading distinguishes three groups identified for read-across purposes.

that was only marginally improved by considering both gastric and intestinal release in a sequential manner ($R = -0.96$, $R^2 = 0.93$, data not shown). Two hours was selected as the most biologically relevant time point when comparing gastric bioaccessibility to *in vivo* acute oral toxicity. Tomlin et al. (1993) reported the average half time for gastric emptying in normal human volunteers to be 17.7 min. Similarly, one study reported a complete gastric emptying time of approximately 91 min in human volunteers (Méndez-Sánchez et al., 2001). The average residence time in the gut has been reported to be 2 h in rats as demonstrated by a study in which gastric emptying of glass beads was found to be approximately 73% at 2 h (Wang et al., 2001).

We also determined that absolute nickel ion release (μg nickel/g sample) or nickel ion release corrected by surface area (for the sparingly soluble compounds) correlated poorly with the observed acute oral toxicity LD_{50}s (data not shown). Therefore, the percent of available nickel released after 2 h in synthetic gastric fluid provided the strongest relationship with the LD_{50} values. Differences between duplicate measurements of percent nickel release were found to be small (<6% of the average of the two values for all but one sample; data not shown) and as such LD_{50} values were examined relative to the average of the duplicate nickel release measurements (Fig. 1). The acute oral toxicity data were found to be well predicted by the nickel release data based on the model: $LD_{50}^{1/2} = 109.2 - 0.9123 \times$ nickel release (% available nickel). The fit and predictive power of the regression line was very good with 91% explained variance ($R^2 = 0.91$). In addition, Fig. 1 indicates that residual (observed–predicted) values are of similar magnitude across the nickel release prediction range, providing confidence in the appropriateness of using normal distribution-based confidence limits for predicted values. This good fit provides sufficient verification that the gastric bioaccessibility data after 2 h can be used to predict acute oral toxicity and to identify source substances from which to perform read-across to target substances.

3.4. Predicting acute toxicity (step 3)

The next step was to determine the extent to which the relationship between bioaccessibility and the *in vivo* data could be used to predict oral LD_{50} values and subsequently be used to group substances for regulatory classification of oral systemic endpoints including acute and chronic oral toxicity and reproductive toxicity. To do this, one-sided lower 95% confidence limits for individual predicted LD_{50} values were constructed using standard least squares regression methodology. Fig. 2 shows the fitted nonlinear relationship between LD_{50} and nickel release with associated lower 95% confidence limits for individual predicted LD_{50} values.

For the purposes of read-across, the substances tested can be divided into three groups by comparing the lower 95% confidence limits for LD_{50} values to the classification cut-offs as a conservative approach (horizontal lines correspond to the 300 and 2000 mg/kg bw cut-off limits for classification specified by the CLP system (EC, 2009b)). The data indicate that substances with nickel release in gastric fluid less than approximately 48% are predicted to exceed an oral LD_{50} of 2000 mg/kg with 95% confidence. This is supported by reliable *in vivo* data where all substances tested in this range (nickel oxide, hydroxide, sulfide and subsulfide) had an LD_{50} of 5000 mg/kg or greater.

A second group was identified at the upper end of the nickel release scale based on the lower 95% confidence limits for LD_{50} values of with substances having >76% bioaccessibility. Substances in this bioaccessibility range would be expected to generally have acute toxicity values below 2000 mg/kg, based on substances in this range tested to date, which have all had reliable LD_{50} values between 300 and 2000 mg/kg (nickel acetate, chloride, fluoride, hydroxycarbonate, sulfamate and sulfate). This included the most water soluble substance that released nickel at a rate of 91% in gastric fluid (nickel sulfate). Consequently, although nickel substances fitting this range could in theory have an LD_{50} below 300 mg/kg, the observed toxicity values indicate they are likely to have an LD_{50} between 300 and 2000 mg/kg. Finally a third potential group of nickel compounds was identified based on the lower 95% confidence limits for LD_{50} values of samples releasing between 48% and 76% nickel in gastric bioelution tests. Any nickel substances with nickel release levels within this range are predicted to have oral LD_{50} values that are higher than 300 mg/kg with 95% confidence but may also exceed 2000 mg/kg in some cases. Since there are no nickel substances tested so far corresponding to this range of bioaccessibility values, further *in vivo* testing would be needed to determine the acute oral toxicity of individual substances in this range.

3.5. Read-across and grouping for CLP classification (step 4)

Using the approach described above, the nickel substances tested can be grouped according to bioaccessibility in gastric fluid.

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Table 5
Summary of CLP hazard classifications for source nickel compounds and target substances that were read across for repeated oral toxicity endpoints.

Source nickel compounds	Existing classification	Oral route-related classifications	Read across oral route-related repeated dose classifications to the following target compounds
Ni sulfate	Acute Tox 4; H302; Acute Tox 4; H332; Skin irrit 2; H315; Skin Sens 1: H317; RespSens 1: H334; STOT RE 1: H372; Muta 2: H341; Carc 1A; H350 inh; Repr. 1B; H360D	Acute Tox 4; H302; Repr. 1B; H360D; no classification for repeated dose oral toxicity	Ni acetate, Ni chloride, Ni sulfamate, Ni hydroxycarbonate, Ni fluoride
Ni oxide	Skin Sens 1: H317; STOT RE 1: H372; Carc 1A; H350 inh	No classifications	Ni hydroxide, Ni sulfide
Ni subsulfide	Skin Sens 1: H317; STOT RE 1: H372; Muta 2: H341; Carc 1A; H350 inh	No classifications	

CLP, European Regulation on Classification, Labelling and Packaging of chemical substances and mixtures.

This grouping approach was used to read-across toxicities to data-poor nickel substances in order to assess hazard classifications using the GHS-CLP system (EC, 2009b). Three source nickel compounds (nickel sulfate hexahydrate, nickel subsulfide and nickel oxide) were identified for which there is a considerable body of toxicological information available and harmonized classifications exist in the EU (EC, 2009a). See Table 5.

3.5.1. Acute oral toxicity

According to the CLP Regulation, the classification criteria for acute oral toxicity divide substances into four categories based on their LD_{50} values (mg/kg bw): Cat. 1 ($\leqslant$5), Cat. 2 (5–50), Cat. 3 (50–300), and Cat. 4 (300–2000). Substances with toxicity values above 2000 mg/kg do not receive a classification for acute oral toxicity. As indicated in Table 4 and Fig. 2, nickel substances have LD_{50} values ranging from 310 mg/kg to greater than 11000 mg/kg fitting the requirements for classification as Category 4 (e.g., source compound nickel sulfate) or no classification for acute toxicity (e.g., source compounds nickel subsulfide and nickel oxide).

3.5.2. Reproductive toxicity

Hazard classification categories for reproductive toxicity are defined in the CLP guidance as Cat. 1, which corresponds to a "known" (Cat. 1a) or "presumed" (Cat. 1b) human reproductive toxicant. Category 2 is assigned to substances considered to be a "suspected human reproductive toxicant". Detailed criteria regarding reproductive toxicity are available in the CLP guidance document (EC, 2009b). Water soluble nickel substances (including the source compound nickel sulfate) are generally classified as Cat. 1b, with the specific hazard statement H360D for developmental effects based on results of an oral study conducted in rats with nickel sulfate hexahydrate (EC, 2009a; SLI, 2000). This study is considered to provide information on soluble nickel compounds as a worst case scenario for all other nickel substances, since these compounds have the highest oral absorption and can result in the highest systemically bioavailable levels of nickel (II) ion. Less soluble nickel substances, such as the source compounds nickel subsulfide and nickel oxide, have shown >20- and >1000-fold lower oral absorption, respectively, than nickel sulfate in rats (Ishimatsu et al., 1995). Consequently, these substances are not expected to release enough nickel ion to exceed the threshold of reproductive toxicity and therefore have not been classified for this endpoint in the EU. In addition, the classification of water soluble nickel compounds based on these animal data should be viewed with caution as the same effects have not been observed in an extensive cohort of female nickel refinery workers (Vaktskjold et al., 2004, 2006, 2007, 2008a, b). Nickel substances have not been associated with fertility effects, and so do not carry any additional designation for this endpoint.

3.5.3. Repeated dose oral toxicity

In addition to acute oral toxicity and reproductive toxicity, repeated dose oral toxicity should also be evaluated when looking at systemic oral toxicity. According to the CLP guidance, substances are evaluated for this endpoint using expert judgment and classified as specific target organ toxicants following repeated exposure based on the weight of evidence of all available data (EC, 2009b). Substances assigned as Category 1 are "presumed to have the potential to produce significant toxicity in humans" based on human data or studies in experimental animals at generally low exposure levels. Category 2 applies to substances that are "presumed to have the potential to be harmful to human health" based on animal studies where generally moderate exposure levels result in effects relevant to humans. However, no nickel substances are classified for chronic oral toxicity as sufficient data exist for water soluble nickel compounds (i.e., worst case scenario for all nickel substances) indicating that no classification is warranted (Heim et al., 2007).

3.5.4. Read-across

Based on the groupings described above and the existing hazard classifications for source nickel compounds, a read-across assessment was implemented for nickel substances registered under the Nickel Consortia for REACH (www.nickelconsortia.org). It should be noted that in all instances, actual toxicity data were used for classification where available (e.g., acute oral toxicity). See Table 5. The data presented in Fig. 2 suggest that three groups can be identified for nickel substances with regards to systemic oral toxicity. First, samples releasing <48% nickel ion in gastric fluid can be read-across from two source compounds (Nickel oxide green and Nickel subsulfide). Based on read across, the substances in this group would carry no classifications for the endpoints of repeated oral and reproductive toxicity. Substances belonging to this group are nickel hydroxide and nickel sulfide (Table 5).

Second, samples releasing >76% of available nickel in gastric fluid were read-across from nickel sulfate. The nickel sulfate sample released the most nickel ion of any sample and thus provides a conservative estimate of potential systemic toxicity. Substances with gastric bioaccessibility between 76% and 100% would warrant classifications for acute oral toxicity (Acute Tox 4) and reproductive toxicity (Repro 1b). These compounds include nickel sulfamate, nickel hydroxycarbonate, nickel acetate and nickel chloride (Table 5).

Finally, a third group could potentially be established for samples releasing 48–76% nickel ion release. It is predicted that such substances would receive an acute oral toxicity classification of Acute Tox. 4 based on the lower confidence interval (predicted LD_{50} > 300 mg/kg with 95% confidence). However, it is possible that *in vivo* testing would demonstrate the LD_{50} is higher than 2000 mg/kg and no classification would be relevant. The data are

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not sufficient to make an assessment regarding longer term endpoints such as reproductive toxicity or repeated dose toxicity for this range of bioaccessibility. Any read-across performed for these endpoints would require additional data to be generated (e.g., toxicokinetic data). Currently, no source or target compounds fall in this range. Should bioaccessibility data for a new compound indicate this group is appropriate, and no verification data exist, then read-across to the more conservative source compound should be performed (e.g., nickel sulfate, see below).

3.6. Implications for classification

Water solubility alone has been used as a basis for grouping nickel compounds for the purposes of read-across for classification. For more than one hundred nickel compounds, water solubility-based classifications without confirmatory *in vivo* data were included in the first ATP to the European Regulation on Classification, Labelling and Packaging of Chemical Substances and Mixtures (CLP; EC, 2009a; b). In comparison to these harmonized classifications, the newly acquired bioaccessibility and acute oral toxicity data for 11 nickel substances suggest that two of those substances (nickel chloride and nickel dihydroxide) should be less stringently classified and one substance (nickel sulfamate) should receive a more stringent classification for systemic endpoints than currently assigned. As discussed in our companion manuscript, the *in vivo* data generated in this study suggest that several changes in acute oral toxicity classification are warranted (Henderson et al., 2012). For nickel chloride, the revised LD_{50} indicates that it should be classified as Acute Tox. 4; H302, a less stringent classification than the existing harmonized classification of Acute Tox. 3; H301. The existing CLP classification of nickel hydroxide as Acute Tox. 4; H302 should be reevaluated, as the data suggest the classification should be eventually removed so that nickel hydroxide no longer carries a classification for acute oral toxicity according to the CLP criteria. Finally, nickel sulfamate currently carries no classification for acute toxicity in the EU, but the outcome of the new study suggests that an Acute Tox. 4; H302 should be applied based on its LD_{50} of 1098 mg/kg.

Read-across based on gastric fluid bioaccessibility data indicates that for one substance (nickel dihydroxide) the current reproductive toxicity Category 1b classification does not apply. The assessment presented here indicates that nickel dihydroxide should be read across from nickel oxide for systemic endpoints. The results indicate that nickel dihydroxide is not likely to release enough nickel (II) ion to result in sufficiently bioavailable levels to exceed the threshold for reproductive toxicity, and hence should not be classified as such. The data support the declassification of nickel dihydroxide as a Repro. Cat. 1b. However, Title V of the CLP Regulation states that if a harmonized classification exists and is in Annex VI of the CLP, this classification must be followed and no self-classification can be done (EC, 2009b). Therefore, despite new assessments that have been made based on this program, the registration files submitted by the Nickel REACH Consortia reflect what has been published in the first ATP to the CLP (EC, 2009a). All suggested classification changes are summarized in Table 5.

4. Conclusions

In order to comply with the data and classification requirements of the new REACH regulatory system in the EU, a research program was undertaken to address data gaps for nickel substances undergoing registration. The results presented within this report summarize the development of a read-across paradigm for nickel substances and systemic toxicities based on gastric bioaccessibility data together with confirmatory *in vivo* animal testing.

The read-across paradigm developed here can be used to extrapolate the systemic toxicity data of source compounds to other nickel compounds. Besides hazard classification, the read-across paradigm presented here can be used to identify the proper source substance from which to read across Derived No Effect Levels (DNELs) for various toxicity endpoints. The grouping based on bioaccessibility can be extended to other divalent inorganic nickel substances as well as some organic substances when sufficient data are available to make a valid assessment. In addition, one key limitation to note is that this paradigm does not address the potential toxicity of any counter-ion that may be present. Therefore, application of these data for read-across to other substances should only be conducted when counter-ion toxicity has been excluded. This requires generation of minimal *in vivo* toxicity data to verify results of bioaccessibility testing. It is important to note that in all cases, data generated from actual *in vivo* toxicity studies supersede any conclusions that could be made based on this read-across assessment. Assessments based on bioaccessibility data must be made for each route of exposure by investigating metal ion release in relevant biological fluids, such as synthetic lung fluids for inhalation and synthetic sweat for dermal. This approach enables a more scientifically valid read-across that can be endpoint- and exposure-route specific, which means that there may be a different source substance identified for each route of exposure. Hence, not all endpoints for one target substance will be read across from the same source substance as is the case when using simple water solubility as bases for extrapolation.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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