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RIVM Letter Report 711701098/2010

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Authors: M.I. Bakker
             E.F.A. Brandon
             J.C. van Eijkeren
             W. Slob

Contact:
Martine I. Bakker
Centre for Substances and Integrated Risk Assessment
martine.bakker@rivm.nl

Abstract

For a more refined risk assessment of lead contamination in Dutch made grounds the bioaccessibility of lead from these soils can be used. Several laboratory models exist for assessing the lead bioaccessibility from soil (*i.e.* lead released from the soil and available for absorption by the gastrointestinal tract of children). In the Netherlands, both the *in vitro* digestion model of the RIVM and the Tiny Tim model of TNO mimic the physiological conditions of the gastrointestinal tract of a child. However, *in vivo* validation of these methods is needed for Dutch made grounds to be able to select the *in vitro* digestion model which shows the best prediction for exposure in a child. In addition, results from an *in vivo* experiment can be used to derive a generic bioavailability factor for lead in Dutch made grounds. An *in vivo* validation study with juvenile swine is proposed, together with an investigation into adjustments of the *in vitro* models, as suggested by international experts.

Key words:
lead, bioavailability, bioaccessibility, soil, *in vitro* digestion, *in vivo*, validation

Rapport in het kort

***In vivo* validatie van *in vitro* modellen voor de orale biobeschikbaarheid van lood uit Nederlandse stedelijke ophooglagen**

Dit rapport beschrijft de opzet voor een validatiestudie met jonge varkens. Deze dieren zijn een goed model voor de opname van lood door kinderen. Tevens wordt beschreven welke experimenten uitgevoerd zullen worden met de laboratoriummodellen (*in vitro* methoden) om tot de beste opzet van deze modellen te komen. De voorkeur gaat ernaar uit via de validatiestudie tot een keuze te komen voor één Nederlands laboratoriummodel dat de situatie voor de mens het beste voorspelt.

De bodem van Nederlandse oude binnensteden (ook wel stedelijke ophooglagen genoemd) is vaak verontreinigd met lood. Kinderen zijn gevoelig voor de toxische effecten van lood en een te hoge bloed-loodconcentratie kan tot een vermindering van het IQ leiden. Er bestaan wereldwijd meerdere laboratoriummodellen die schatten hoeveel lood uit de bodem vrijkomt in het maagdarmkanaal van kinderen en hoe groot de interne blootstelling zal zijn. Deze modellen bootsen de condities van het maagdarmkanaal van een kind na. In Nederland zijn dit het IVD-model van het RIVM en het tiny TIM-model van TNO.

Een validatie van deze laboratoriummodellen met de werkelijke interne blootstelling in kinderen is van groot belang, omdat de informatie die met de laboratoriummodellen verkregen wordt voor een meer verfijnde risicoschatting gebruikt wordt. Het is daarom belangrijk om te weten of deze laboratoriummodellen een goede schatting, of een onder- of overschatting geven van de interne loodblootstelling na inname van bodem door kinderen. Met behulp van de validatiestudie kan waarschijnlijk een generieke interne blootstellingswaarde afgeleid worden voor Nederlandse stedelijke ophooglagen.

Trefwoorden:
lood, biobeschikbaarheid, bioaccessibility, bodem, *in vitro* digestie, *in vivo*, validatie

Contents

1   Introduction

In The Netherlands two *in vitro* models are in use that can simulate the digestion of substances, namely the 'Tiny' TNO *In vitro* Model (TIM) and the RIVM *In Vitro* digestion (IVD) method. Both methods can be applied to estimate the humane bioavailability of lead in soil matrices. To investigate if both methods would yield comparable results, the bioaccessibility of lead in soil samples from sixteen Dutch made grounds was determined with the two methods (Hagens *et al.* 2009). The results of the two models appeared to be very different: the bioaccessibility of lead according to the Tiny TIM model was a factor 4-5 lower than that of the IVD model.

Five international experts have reviewed this research of the two methods. In addition, they have given their opinion on which of the models could be used in the practice of risk assessment. The reviews were discussed during a workshop in May 2009. It was concluded from the workshop that:

- The different results of the two methods were likely mainly caused by the pH of the gastric phase and the different separation techniques (ultra-filtration versus centrifugation).
- *In vivo* validation is required for a responsible application of the *in vitro* models for risk assessment of lead in soils.
- This should be done with samples of Dutch made grounds, preferably using young swine.
- The model showing the highest correlation with the *in vivo* data (for a large bioavailability range) will be considered the best applicable model.
- In addition, the selected model should be: 1) simple (feasible to operate in routine application at more than one location), 2) responsive to different lead and soil characteristics, 3) accompanied by rigorous Quality Assurance/Quality Control data requirements (*e.g.* with regard to recoveries, blanks, reproducibility).
- For both methods, most likely adaptations to the current methods will be needed, *e.g.* with respect to the amount of soil used.
- For the IVD-method simplification of the model (to only the gastric phase) should be considered.

The main objective of the *in vivo* validation study is the validation of the two *in vitro* models. With the results of this study the best predictive *in vitro* model can be selected. The selected model can be applied in the site-specific risk assessment of lead in Dutch made grounds.

The current report presents a proposal for an investigation into adjustments of the *in vitro* models, suggested during the workshop (Chapter 2). These adjustments should lead to more comparable results for the different models. In addition, the report addresses *in vivo* validation studies with juvenile swine (Chapter 3) and the proposed set-up for the *in vitro* and the *in vivo* validation study for Dutch made grounds (Chapter 4). In the final chapter an overview of the estimated costs is presented.

2 Future developments in bioaccessibility testing

During the workshop the experimental design of the bioaccessibility testing of lead from Dutch made grounds with the two *in vitro* models were discussed. In this chapter the further development of the *in vitro* models, as suggested by the international experts, is proposed.

2.1 Soil sampling and characterisation

2.1.1 Particle size fraction

In the study of Hagens *et al.* (2009) on the bioaccessibility of lead of Dutch made grounds, the samples, after sieving and removal of large parts, were "smashed" to 2 mm. The < 2 mm fraction is standard practice in soil and agricultural science, whereas in bioaccessibility studies a fraction < 250 µm is normally used. It has been suggested that the smaller particles stick more to children's hands. Therefore, it would be reasonable to propose using the < 250 µm fraction for the bioaccessibility experiments and for the determination of the total lead concentration in the soil. However, in the Dutch legislation maximum tolerable levels are expressed as concentrations of lead in the soil of < 2 mm. So, for policy purposes, information on the bioavailability of this particle size fraction is required rather than for the < 250 µm fraction. Inclusion of another size fraction in the risk assessment would increase the complexity, also because the total lead concentration will differ between the different particle size fractions. Hence we propose to use the same particle size as in the previous study, namely < 2 mm. In addition, it is proposed to also collect the < 250 µm fraction to enable international cooperation, *e.g.* to validate the Unified BARGE Method (UBM).

Proposal: Use the particle size fraction of soil samples of <2 mm with the IVD and Tiny TIM model for validation of the Dutch situation and <250 µm for international cooperation.

2.1.2 Analysis of total lead

In The Netherlands the total lead concentration of soil is normally determined by extraction with *aqua regia*. However, in the study of Hagens *et al.* (2009) there appeared to be considerable variation between the total lead concentrations of the made ground samples between the different laboratories. This is likely caused by the fact that slight differences between the implementation of the extraction protocol may lead to a considerable difference in total lead because not all lead is liberated from the soil by *aqua regia*. An alternative reagent for the extraction of lead from the soil, proposed by one of the reviewers of Hagens *et al.* (2009) would be hydrogen fluoride, which is more effective in dissolving all lead from the soil particles. Hydrogen fluoride is used for this purpose in *e.g.* Canada. However, in the Netherlands the risk assessment of lead in soil is based on the extraction method with *aqua regia*. For this reason, the amount of total lead extracted with hydrogen fluoride is not useful. Therefore, we conclude that in the bioaccessibility studies in the Netherlands, *aqua regia* is the best extraction fluid.

Nevertheless, the current protocols for *aqua regia* extraction need to be adapted to make them stricter. Stricter protocols may prevent variation between the results of the different laboratories and so ensure a better reproducibility of the total lead determination.

The protocols for extraction with *aqua regia*, developed by the foundation Infrastructuur Kwaliteitsbeheer (SIKB), are available at the Dutch Normalization Institute (NNI). A study into the protocols is needed to answer the following questions: How much variation is allowed? And do the results of RIVM and TNO of the study of Hagens *et al.* (2009) fit within these margins? The use of *aqua regia* will lead to a lower total-lead determination than the use of hydrogen fluoride. This may not be a problem, as the part of the lead that is not dissolved in *aqua regia* may not be bioavailable either. However, the *aqua regia* method needs to be very strictly described and the regulations should be strictly followed. The study into the protocols, leading to stricter protocols, is not part of the study described in the present proposal.

In addition to the analyses of total lead with *aqua regia*, it is proposed to extract the samples with nitric acid. Nitric acid extractions (0.43 M) of soil can be used to predict the free concentration of metals in the soil, which is related to the bioaccessibility of the metals in the soil (Dijkstra *et al.* 2009, Groenenberg *et al.* 2010). This is in contrast with *aqua regia* which also extracts non-bioavailable parts of the metals from the soil.

The British Geochemical Survey (BGS, Mark Cave) has offered to determine the total lead concentrations in the soil samples with X-Ray Fluorescence (XRF). In this way the extractions with *aqua regia* could be validated.

The total lead concentrations will be determined for the < 2mm and < 250 μm particle size fractions.

Proposal: *In our lead bioaccessibility studies the total concentration of lead will be determined by extraction with aqua regia, but the protocols need to be adapted so that less variance is obtained. The adaptation of the protocols is not part of the present study. Validation of the aqua regia extraction can be performed by analysis of the soil samples with XRF by BGS. In addition, it is proposed to extract the samples with 0.43 M nitric acid. We propose that a sufficient amount of soil material will remain available after the in vivo experiments for other researchers to study the relationship between bioaccessibility and soil characteristics.*

2.1.3   Lead characterisation

In the study of Hagens *et al.* (2009) three methods were used to characterise the lead pollution in the made ground samples: First, multi-element analysis in combination with a statistical cluster analysis was used. Secondly, lead isotope analysis was performed to further characterise the soil samples and determine in more detail which lead source is responsible for the lead pollution in the soil. Finally, Scanning Electronic Microscopy (SEM) was used for a sub-selection of soils to study the chemical compositions and particle sizes (diameter) of the anthropogenic lead phases (both primary and secondary) present in these made

grounds. With the SEM pictures a PPS (Primary lead phases, Particle size and Secondary lead phases) ranking was established for the 16 soils.

In the reviews of the report of Hagens *et al.* (2009) and the following workshop it was suggested that these three techniques were not sufficient for a thorough characterization of the samples. It was concluded that more quantitative characterization of representative samples of the Dutch made grounds would be a worthwhile investment to improve on the PPS ranking. (Bakker and Hagens, 2009)

The following suggestions for additional characterization of made grounds were made during the workshop:
- chemical speciation: inorganic and organic lead. Techniques to identify fine-grained lead-bearing compounds: (XANES, micro XRD, micro XRF, FTIR);
- particle size distribution of the different soils.

However, as the aim of the validation study is to validate the *in vitro* model and not to find correlations between the soil and lead characterisation, we propose to perform no additional characterisation of the made ground samples. Furthermore, we propose to omit the SEM-analyses as these are very costly and they give relatively little new information since we want to test soils that are analysed by SEM before.

Proposal: Lead characterisation of the soil samples will be done with multi-element analysis and lead isotope analysis. With respect to soil characterisation we propose to analyse the same parameters as in the previous study (lutum, organic matter, $CaCO_3$, pH).

2.2   *In vitro* models

2.2.1   *In vitro* digestion model (IVD)

Sample size and reproducibility

The solid to fluid ratio of 1:1000 mimics the hand-to-mouth behaviour intake of a child. In the IVD model this ratio corresponds to 0.06 g of soil. A correlation line of 1.16 was observed for the *in vitro-in vivo* correlation of Bunker Hill soil using 0.06 g soil, indicating a slight overestimation of the actual risk. In the same study, 0.6 g of soil per digestion led to an *in vitro-in vivo* correlation slope of 0.69, indicating an underestimation of the bioavailability. These results indicate that an amount of 0.06 g results in a better measure of bioaccessibility and thus in a better input for risk assessment. However, 0.06 g is a very small amount and it may be questioned if this is sufficient for obtaining a reproducible bioaccessibility value (Bakker and Hagens, 2009).
In addition, a significant lower bioaccessibility value for lead was observed under fasted conditions when 0.6 g of Montana soil 2710 was used compared to 0.06 g soil. A mean bioaccessibility of 23 ± 4% (n = 14) versus 43 ± 9 % (n = 23) was observed, resulting in a relative standard deviation of 17 and 21 %, for 0.6 g

and 0.06 g soil per digestion tube, respectively. Although this indicates a good reproducibility for the amount of 0.06 g, Montana soil 2710 is a fine and homogenous soil. In contrast, inhomogeneous soils, *e.g.* Dutch made grounds, could lead to a lower reproducibility. Therefore the bioaccessibility reproducibility with the IVD model of Dutch made grounds for the amount of 0.06 g should be tested. The reproducibility of the bioaccessibility under fed conditions was determined for Montana Soil in only a few experiments. Therefore, the reproducibility of the IVD-method under fed conditions is not known and needs to be determined in future studies.

If the reproducibility of the IVD model with made ground samples of 0.06 g is not sufficient (*i.e.* not comparable with samples of 0.6 g), the reproducibility may be improved by using a soil-to-fluid ratio of 1:1000 in combination with a larger amount of soil and upscaled fluid volumes. For instance, for an amount of 0.2 g of soil, this would mean a total volume of ~200 ml of chyme.

Proposal: In a preliminary experiment, the reproducibility of the IVD-model with normal fluid volumes will be studied with 0.06 g and 0.6 g for three Dutch made ground samples. In addition, 0.2 g of soil with upscaled fluid volumes (1:1000 soil-to-fluid ratio) is investigated for its reproducibility. An amount of 0.06 g is used in future IVD experiments if the reproducibility is comparable to or better than 0.2 and 0.6 g. Otherwise, the upscaled system with 0.2 g soil per digestion tube is used for further IVD experiments.

Separation method

Different methods can be used to separate the chyme from the fraction that is too large to be absorbed and thus to determine the bioaccessible fraction of lead after *in vitro* digestion. Centrifugation, ultra-filtration and micro-filtration are techniques that have been used to separate the chyme from the non-absorbable fraction. Currently, centrifugation is used in the IVD model to separate the chyme and pellet. In contrast, the US EPA uses micro-filtration (0.45 μm cellulose acetate disk filter) to separate the absorbable fraction from the non-absorbable fraction. Data from a previous study showed that the separation method (centrifugation, ultra-filtration and micro-filtration) substantially influenced the bioaccessibility, indicating that the method of separating digestive fluid from the digested soil affects the input for risk assessment and the *in vitro-in vivo* relationship. However, these conclusions are only based on one soil and under fasted conditions. Further research on the method of separation is recommended, especially for fed conditions, since it is expected that the presence of food in the model (see section 2.2.2) will clog the filters of the micro-filtration and especially the ultra-filtration separation method. An additional filtration step may be necessary, which will reduce the reproducibility of the model.

Proposal: Two separation techniques (centrifugation and ultra-filtration) will be investigated in a preliminary experiment to study the influence of the separation method on the bioaccessibility of lead and to assess whether it is technically possible to use filters for simulated semi-fed conditions.

Fasted versus fed conditions

The bioaccessibility of lead from soil is determined from the intestinal phase, but has also been determined in the stomach. The bioaccessibility of lead from 0.06 g of Montana soil 2710 is 78 ± 9 % (n = 11) in the

stomach and 43 ± 9 % (n = 23) in the small intestine. In addition, a bioaccessibility of 64 ± 4% (n = 11) in the stomach and 23 ± 4% (n = 14) in the small intestine was observed for 0.6 g of Montana soil 2710. So, the bioaccessibility is higher in the stomach compartment than in the intestinal compartment. Absorption of lead only occurs in the small intestine, thus using the stomach bioaccessibility in risk assessment would most likely lead to an overestimation of the risk. In the *in vivo* correlation study the bioaccessibility of lead from both the stomach and intestinal compartment should be investigated and the correlation with the *in vivo* data should be determined. Based on these results a method should be chosen for future bioaccessibility research.

The *in vitro* digestion model simulates fasted conditions as a default, because lead is better absorbed and more bioavailable under fasted conditions than under fed conditions (James et al., 1985; Heard et al., 1982; Heard et al., 1983; Blake et al., 1983). Indeed, the bioaccessibility of lead from soil is highest under fasted conditions (Hagens et al., 2008; Oomen et al., 2006; Lijzen et al., 2006). Nevertheless, exposure to lead via soil is assumed to be a chronic process and children will not always be completely fed or fasted when contact with soil occurs (Oomen *et al.*, 2006). Thus, a bioaccessibility from an "average physiological state" is more realistic in risk assessment than fasted conditions or fed conditions (Hagens *et al.*, 2008). Therefore we propose to simulate this average physiological state both in the *in vitro* and *in vivo* models, using semi-fed conditions[1]. A pH of 2 and 1 g of food (under fully fed conditions a pH of 2.5 and 2 g of food is used) using normal fluid volumes is proposed.

Proposal: The experiments with IVD (the preliminary experiments as well as the validation experiment) are performed under semi-fed conditions. In the validation experiment both the gastric and the intestinal phase will be analysed.

2.2.2   Tiny TIM

Amount of soil

The previous bioaccessibility experiments with soil in Tiny TIM were performed with 5 g of soil, to mimic pica (soil-fluid ratio 1:100). Nevertheless, based on a decision of policy makers, in the present validation experiment hand-to-mouth behaviour will be studied (soil-fluid ratio 1:1000). The influence of the amount of soil will be investigated in a preliminary experiment with Tiny TIM. In addition to 5 g of soil, an amount of 0.5 g, corresponding with a soil-fluid ration of 1:1000, will be tested.

Proposal: In a preliminary experiment with Tiny TIM, the bioaccessibility of lead from soil will be measured with amounts of 0.5 g and 5 g soil.

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[1] The IVD model stomach compartment applies a pH 1.5 ± 0.5 under fasted conditions and a pH of 2.5 ± 0.5 under fed conditions Under (semi-)fed conditions the bioaccessibility of lead from soil is studied using the *in vitro* digestion model in combination with infant food. Infant food Olvarit 15M52 (leek, mushrooms with ham and carrots) (Nutricia®, The Netherlands) supplemented with 2 g sunflower oil per 100 g is used and simulates the standard Dutch dinner based on the macronutrients and caloric composition in the third Dutch National Food Consumption Survey of 1998 (Kistemaker *et al.*, 1998). Infant food is made commercially and of constant composition and can therefore be used as food source for the *in vitro* digestion model under fed conditions.

Lower pH in gastric phase

In tiny TIM experiments with soil, the gastric pH decreases from 5 to 2 in 90 minutes, whereas in IVD under semi-fed conditions a value of 2 is maintained for 2 hours. In the workshop, there was agreement that in tiny TIM a longer 'exposure' to a lower pH in gastric phase is needed. Therefore, in a preliminary experiment the influence of a lower pH (pH of 2 in the first 90 minutes) will be investigated.

Proposal: In a preliminary experiment with Tiny TIM, the influence of the pH will be investigated using a pH of 2 during the first 90 minutes.

Separation method

The separation method normally used in Tiny TIM is ultra-filtration: a membrane unit is connected to the intestinal compartment for the continuous removal of released and digested small molecule weight (MW) compounds (< 10 kD) and water. To test the influence of the separation method on the bioaccessibility of lead, the two different separation techniques (ultra-filtration and centrifugation) will be tested in a preliminary experiment with Tiny TIM, similar to the experiment described above for IVD. As the experiments in Tiny TIM are performed under simulation of the average physiological conditions without adding food, problems with clogging of the filters are not to be expected.

Proposal: Two separation techniques (centrifugation and ultra-filtration) will be investigated in a preliminary experiment with Tiny TIM to investigate the influence of the separation method on the bioaccessibility of lead.

3 *In vivo* studies into bioavailability of lead

3.1 Human studies versus animal models

Currently, the bioavailability of lead from soil was determined in one human volunteer study. This study investigated the bioavailability of lead from Bunker Hill soil (<250 µm fraction) in adults under both fasted and fed conditions (Maddaloni *et al.*, 1998) by stable isotope dilution. The investigated soil was from a residential yard at a mining-impacted federal Superfund site that had negligible amounts of other priority pollutants and had a mean soil lead concentration of about 2900 mg/kg. The bioavailability of lead from soil was 26 ± 8 % for fasted conditions (without breakfast) and 3 ± 2 % for fed conditions (with a standardised breakfast).

Human volunteer studies investigating the bioavailability of lead are not ethically accepted, especially not in children. Therefore, other *in vivo* validation studies are needed to validate the IVD model for lead in children.

Animal species, especially rat, have been used to investigate the lead bioavailability in humans. However, based on physiology and comparison of other substances than lead, rats and these other species are found to be a poor model for human bioavailability of lead. Only juvenile swine have shown to be a good model for the bioavailability of lead in children by the U.S. EPA (U.S. EPA 2006; Weis *et al.*, 1994). Therefore, no further research was performed to investigate other species as a method to validate IVD and Tiny TIM for lead in children.

3.2 University Missouri Juvenile swine model

The group of professor Casteel at the University of Missouri has much experience in the specific subject of bioavailability of metals in juvenile swine and validation experiments of the US-EPA *in vitro* bioaccessibility method were performed. Below, the method for juvenile swine as performed by professor Casteel and co-workers is described.

In the juvenile swine model of the University of Missouri the *in vivo* relative bioavailability of lead in soil is measured (Casteel *et al.*, 2006). The term 'relative' refers to relative to lead acetate solution, which is used as a reference material. The lead acetate or lead-contaminated soils are administered orally to juvenile swine (male pigs weighing 10-12 kg, 4-5 animals in a dose group, 3 dose groups per test material) twice a day for 15 days. Feeding occurs two hours after dosing, hence the condition of the swine during administration is semi-fasted. Blood samples are collected from each animal at multiple times during the course of the study, and samples of liver, kidney, and bone are collected on the last day of dosing. All

samples are analysed for lead. In the *in vivo* studies, toxicity to the swine is not observed (Casteel *et al.* 2006).

The reproducibility of the relative bioavailability determined with this *in vivo* method is very good: bioaccessibility values of a specific soil of 73 and 75% were measured intra-lab and values of 77 versus 74% between labs. The swine are an out-bred commercial crossbred that mimics the human population as much as possible. Total recoveries of the lead fed to the swine are not determined (because of excretion via faeces). Recoveries from spiked samples are used for the different organs.

The calculation of the relative bioavailability in this method, the measured values of the 19 test soils of the US-EPA study and the costs of this set-up are presented in Appendix 1.

3.3   Alternative *in vivo* validation study

In addition to the method for validation by professor Casteel, we present an alternative study design for the validation of the *in vitro* models. It may be possible to design and perform validation studies in the Netherlands at lower costs, due to the use of kinetic modelling and the availability of lower detection limits.

The alternative *in vivo* validation study we propose is based on the juvenile swine US-EPA study design by professor Casteel (Appendix 1). The alternative design has six animals per test soil and only one dose per soil (whereas the US-EPA study had fifteen animals per test soil divided over three dose groups) and two compartments in which lead is determined (blood and femur, while in the US-EPA design also kidney and liver are measured). For an overview of the parameters of both study designs see Appendix 2. The alternative *in vivo* validation study is proposed to present a study design with reduced costs but with a reliable outcome. The compartments blood and femur were chosen because these are the most relevant ones. To test the variability in the AUCs and femur concentrations of this alternative study design a small pilot experiment will be conducted. The alternative study design is described in more detail in Chapter 4.

3.4   Link to risk assessment

From the *in vivo* experiments with test soils (Test Material, TM), the concentration of lead in the blood (in fact, the area under the concentration–time curve, the AUC) and in the femur is determined and expressed as the concentration in this compartment divided by the given dose (D). The same is done for the experiment with lead acetate (Reference Material, RM). In this way for each test soil the relative bioavailability (RBA) of lead from the test soil is obtained with respect to that of lead acetate, both for the blood and the femur compartment (equation 1).

$$RBA_{TM} = \frac{AUC_{TM,oral}/D}{AUC_{RM,oral}/D} = \frac{femur\,conc._{TM,oral}/D}{femur\,conc._{RM,oral}/D} \qquad (1)$$

However, a value of 'bioavailability relative to lead acetate' cannot be used in the risk assessment of soils, since for risk assessment purposes of soils the absolute bioavailability (ABA) of lead is needed[2]. To obtain the ABA, the absolute bioavailability of orally dosed lead acetate needs to be determined, by using an extra group of swine which are intravenously dosed. By dividing the AUCs (or amounts in femur) of orally and intravenously dosed swine (at the same dose), the absolute bioavailability of lead acetate can be calculated (equation 2). From earlier experiments it is known that typical values for the absolute bioavailability of lead acetate are ca. 15 % (US EPA, 2007).

$$ABA_{RM} = \frac{AUC_{RM,oral}/D}{AUC_{RM,i.v}/D} \ or \ \frac{femur\ conc._{RM,oral}/D}{femur\ conc._{RM,i.v.}/D} \tag{2}$$

Knowing the absolute bioavailability of lead acetate, the absolute bioavailability of lead from the test soils can be calculated from the relative bioavailability that is measured in the experiments by multiplying the BA of the soil relative to lead acetate with the absolute BA of lead acetate (equation 3).

$$ABA_{TM} = RBA_{TM} \times ABA_{RM} \tag{3}$$

3.5 Research groups for *in vivo* experiments with juvenile swine

Validation experiments of the IVD and Tiny TIM model for Dutch made grounds with juvenile swine can be performed at different research groups. The University of Missouri, Columbia, Missouri, USA was already mentioned above. In addition we selected two laboratories within the Netherlands:

- Central Laboratory Animals Institute, Utrecht University, Utrecht, the Netherlands;
- Animal Sciences Group, Wageningen University, Lelystad, the Netherlands.

Central Laboratory Animals Institute
The Central Laboratory Animals Institute (GDL) is a research organisation in research involving animals, including pig. It is part of Utrecht University. Contract research at GDL is carried out according to ISO 9001. However, GDL communicated that they do not have experience with this kind of experiments nor do they have the required animal capacity.

Animal Sciences Group
The Animal Sciences Group is a research organisation in the field of animal production and animal health. The Animal Sciences Group is part of Wageningen University. Contract research at the Animal Sciences Group is carried out according to ISO 9001 and ISO 17025 applies for analyses. ASG provided an offer for

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[2] For comparison with Dutch soil standards, the absolute bioavailability of lead from soil is recalculated into the bioavailability of lead from soil relative to that from food, by multiplying with the fraction of lead from soil that is absorbed (assumed to be 0.8) and dividing by the bioavailability of dietary lead (assumed to be 0.4). This is because the Dutch health limits were based on studies in which animals were dosed with contaminated feed.

performing the *in vivo* validation study for Dutch made ground and can perform these experiments when needed.

Proposal: We propose the alternative study design because it is considered a reliable method to perform bioavailability measurements, against reduced costs. The University of Missouri and ASG are both suitable to carry out this design, because of their long experience with this type of studies.

4 Validation study design

4.1 Dutch made ground samples

A total of 7 locations will be selected for sampling of Dutch made grounds contaminated with lead above the legal limits. The selection is based on the following criteria (ranked from high to low importance):

- bioaccessibility as previously determined: a wide range should be covered.
- different lead characteristics: most information from PPS ranking, but only 16 soils have been ranked.
- different soil characteristics: pH, percentage organic matter, lime and clay
- different soil types: fluviatile sand/clay, aeolian sands, marine sand/clay, dune sand, and loess should all be covered.

The selection of the current locations is a draft proposal (Table 2). A final selection will be made together with Geoconnect in 2010.

Table 2. Draft proposal for location of sampling for the *in vivo* validation study.

nr	location	RBA[1]	soil type	PPS	pH	lime	Organic matter	clay	Useful-ness
1	De Rijp	42	Marine sand / clay	2	7	2	10	7	+++
2	Nijmegen	40	Aeolian sands	4	8	11	3	2	+++
3	Groningen	103	Aeolian sands	6	6	1	22	9	+++
4	Utrecht	11	Fluviatile sand / clay		7	1	1	8	+++
5	Echt-Susteren	64	Loess	4	7	1	2	20	++
6	Leiden	68	Fluviatile sand / clay	5	7	2	5	3	++
7	Den Haag	87	Dune sand	6	7	1	3	1	++

[1] Relative Bioaccessibility (relative to that of food), determined with the IVD-method with average physiological state.

4.2 Sampling

The amount of soil material sampled will be 50 kg (wet weight). This amount is sufficient for the present experiment and future use (*e.g.* for use by BARGE to perform *in vivo* validation studies with other *in vitro* digestion models). The samples will be divided for the lead and soil characterisation, *in vitro* (<2 mm and <250 μm) and *in vivo* (<2 mm) experiments. The sample preparation and the lead and soil characterisation will be performed as previously described by Hagens *et al.* (2009), with two exceptions: 1) the SEM-analysis will be cancelled and 2) an extraction of the soils with 0.43 M nitric acid will be performed.

4.3 *In vitro* experiments

IVD method
The bioaccessibility of different soil samples will be investigated with IVD under semi-fed conditions (with added food), in quadruplicate. Separation of chyme and pellet will be performed by two different methods: ultra-filtration and centrifugation. The chyme produced with the different separation methods will be analysed to determine the lead bioaccessibility. The bioaccessibility of lead (%) is calculated using the following equation: (amount in chyme/amount in soil) × 100%.

Tiny TIM method
The bioaccessibility of different soil samples will be investigated with Tiny TIM under semi-fed conditions (but without adding food), in duplicate. Two separation techniques will be used, namely ultra-filtration and centrifugation[2].
The bioaccessibility of lead (%) is calculated as: (amount in ultrafiltrated or centrifugated efflux of duodenum/amount in soil) × 100%. The calculated bioaccessibility of tiny TIM can be directly compared to that of the IVD-method. Additional tests with lead-acetate will be performed with tiny TIM to compare the results with the *in vivo* studies.

Unified BARGE method
The Unified BARGE method (UBM) is based on the IVD-method and is a harmonised-within-Europe *in vitro* bioaccessibility method. The UBM differs from the IVD-method in the solid to liquid ratio of 1:100 (instead of 1: 1000) and in a lower stomach pH. We propose to validate this method in the present study in addition to the validation of the IVD and Tiny TIM methods, using the < 250 μm fraction, under semi-fed conditions (without adding food, pH of 2, four runs per soil sample). In the UBM, the bioaccessibility is calculated in the same manner as is done in the IVD-method.

4.4 *In vivo* study

The alternative *in vivo* study design will be carried out (Section 3.3). The *in vivo* experiments will be performed with male pigs of 1-1.5 months of age which will be housed individually in lead-free cages and fed low-lead feed. The doses will be administered by adding the contaminated soil to small balls of dough, while the pigs are semi-fed. The pigs will be exposed for 7 days (all doses will be delivered twice daily), after which a 7 day elimination period is introduced. One blood sample will be drawn from each animal each day. Animal weights will be recorded daily. On study day 14 (7 days of uptake + 7 days of elimination), pigs will be humanely sacrificed and a representative sample of bone (right femur) will be collected and prepared for lead analysis.
Firstly, a pilot experiment will be performed with six different doses of lead acetate (ranging from 0 -negative control- to 500 μg/kg/d; one animal per dose) to obtain the variability of the AUCs and that of the lead concentrations in the femur. This is important, since although this may be determined from earlier experiments by the group of professor Casteel, our experiment is set up differently (7 days exposure and 7 days elimination instead of 15 days exposure). In all *in vivo* experiments, calculation of the AUCs and the

body weights of the pigs will take place using a one compartment kinetic model. We consider it likely that we can obtain a lower variability due to the kinetic modelling and to lower detection limits.

The set-up of the final validation experiment is similar to that of the pilot, but will be adjusted if the results of the pilot experiment will indicate the need to do so. In the final validation experiment, there is one dose group (6 animals) for each test soil and one for orally dosed lead acetate that will be used as a reference (Table 2). A relatively low dose will be chosen, using the results of the pilot experiment in combination with those of the *in vitro* experiments with test soils, hence, the non-linear dose-dependency of the AUCs will not occur.

In addition to the negative control, two additional dose groups are needed. To determine the fraction of the total bioavailable lead that is recovered in the blood and femur, one dose group receives lead acetate intravenously (Table 2). As the IV cannula can only be used for a maximum of 5 days, due to skin irritation, an additional, 5-day oral dose group for lead acetate is needed to be able to make a good comparison of the oral and IV dosing (Table 2). From the 5-day experiment, the ratio of the AUCs (and also of the femur concentrations) of the oral and IV dosing with lead acetate can be determined. Using this ratio and the oral 7-day experiment with lead, the bioavailability of lead acetate in the IV dosed swine in the 7 day-experiment can be calculated. This is the value needed to link the results of the *in vivo* experiment to risk assessment, as explained in section 3.3.

A total number of 70 pigs are needed to perform the *in vivo* validation experiment (pilot: 6 + final experiment: 64) with Dutch made grounds.

Table 2. Design for *in vivo* experiment to assess bioaccessibility of lead from soil using juvenile swine.

Group	Number of animals	Treatment	Dose[1]
pilot	6	Negative control + 5 doses of soil	0-500 µg/kg/d
1	4	negative control	oral vehicle
2	6	PbAc (IV; 5 days[2])	weight adjusted
3	6	PbAc (PO; 5 days[3])	weight adjusted
4	6	PbAc (PO; 7 days)	weight adjusted
5	6	soil 1 (PO; 7 days)	mass & weight adjusted
6	6	soil 2 (PO; 7 days)	mass & weight adjusted
7	6	soil 3 (PO; 7 days)	mass & weight adjusted
8	6	soil 4 (PO; 7 days)	mass & weight adjusted
9	6	soil 5 (PO; 7 days)	mass & weight adjusted
10	6	soil 6 (PO; 7 days)	mass & weight adjusted
11	6	soil 7 (PO; 7 days)	mass & weight adjusted

[1] One dose per reference or test material will be used.
[2] Five days is the maximum for IV-dosing, due to irritation of the skin by the cannulae.
[3] Because the IV dosing can only be done for 5 days, for comparison a PO-group is needed which are dosed for 5 days as well.

5 Estimated costs

5.1 Sampling and preparation of Dutch made grounds

A total of 7 Dutch made grounds are included and an amount of 50 kg is taken to prepare the <2 mm and <250 μm fraction used in the *in vitro* and *in vivo* studies.

- Sampling   € 3,850
- Determination of soil and lead characteristics   € 5,890
- Total lead determination   € 2,800
- GeoConnect (coordination and reporting)   € 9,060
- RIVM (coordination)   € 1,500

Total costs sampling and preparation: **€23,100**

5.2 *In vitro* experiments

5.2.1 IVD-model

<u>Preliminary experiment:</u>
- 3 soil samples
    - √ amount of soil 0.06, 0.6 g with normal fluid volumes and 0.2 g with upscaled fluid volumes
    - √ semi-fed (pH 2, 1 g food)
    - √ in ten-fold
    - √ split chyme of samples for testing separation methods
        - centrifugation
        - ultra-filtration

Validation experiment:
- 7 soil samples with soil fraction <2 mm
    - √ semi-fed conditions
    - √ two separation methods: ultra-filtration and centrifugation
    - √ in four-fold
- 7 soil samples with soil fraction <250 μm
    - √ Unified BARGE Method
    - √ in four-fold

Estimated costs
- o *preliminary experiment*   *€ 9,500*
- o *validation experiment IVD*   *€ 8,000*
- o *validation experiment UBM*   *€ 3,000*
- o *Lead analysis:*   *€ 12,500*

5.2.2   Tiny-TIM

Preliminary experiment:
- 3 soil samples
    - √ amount of soil 0.5 and 5 g (2 runs per soil sample)
    - √ amount of soil 0.5 g in combination with gastric pH 2 (1 run per soil sample)
    - √ split chyme of samples for testing separation methods
        - ▪ centrifugation
        - ▪ ultra-filtration

Validation experiment:
- 7 soil samples
    - √ average physiological state
    - √ two separation methods: ultra-filtration and centrifugation
    - √ 2 runs per soil sample

Estimated costs:
- o *preliminary experiment*   *€ 19,000*
- o *validation experiment*   *€ 16,000*
- o *Lead analysis:*   *€ 3,000*

Total cost:
- o *IVD & UBM model*   *€ 33,000*
- o *Tiny-TIM model*   *€ 38,000*
- o *RIVM (coordination and reporting)*   *€ 20,000*
- **Total costs in vitro experiments**   **€91,000**

5.3 *In vivo* experiments

RIVM 'alternative' method:
- 7 days exposure and 7 day wash-out period
- blood (AUC), and bone

- pilot experiment with 6 concentrations (one animal per dose) for lead acetate PO
- final experiment
    - negative control, 4 swine
    - 6 swine, one concentration, for lead acetate, IV and PO
    - 6 swine, one concentration per soil
    - 7 soil samples

Estimated costs:
o *in vivo experiment*	*€ 130,000*
o *RIVM (coordination and calculation)*	*€ 20,000*

Total costs *in vivo* experiments: **€150,000**

5.4 Reporting

The *in vitro* and *in vivo* data and the *in vitro-in vivo* correlation will be reported in an article or RIVM report.

Total costs reporting: **€45,000**

5.5 Overview of the cost for the *in vivo* validation

The overall cost for the *in vivo* validation study for Dutch made ground are summarised in Table 3.

Table 3. Costs *in vivo* validation study

subject	costs
sampling and preparation	€ 23,100
in vitro experiments	€ 91,000
in vivo experiments	€ 150,000
reporting	€ 45,000
total costs	*€ 309.100*

Acknowledgment

References

Bakker MI and Hagens WI (2009). Proceedings of the workshop "In vitro modeling of humane bioavailability of lead from soils". Letter Report 711701094, National Institute for Public Health and the Environment, Bilthoven, the Netherlands.

Casteel SW, Weis CP, Henningsen GM, Brattin WJ (2006). Estimation of relative bioavailability of lead in soil and soil-like materials using young Swine. Environ. Health Perspect. 114 (8): 1162-1171.

Dijkstra JJ, Meeussen JL and Comans RNJ (2009) Evaluation of a Generic Multisurface
Sorption Model for Inorganic Soil Contaminants. Environ. Sci. Technol. 43: 6196-6201.

Groenenberg JE, Römkens PFAR, Comans RNJ, Luster J, Pampura T, Schotbolt L, Tipping E and De Vries W (2010) Transfer functions for solid-solution partitioning of cadmium, copper, nickel, lead and zinc in soils: derivation of relationships for free metal ion activities and validation with independent data, Eur. J. Soil Sci. 61: 58–73.

Hagens WI, Lijzen JPA, Sips AJAM, Oomen AG (2008). The bioaccessibility and relative bioavailability of lead from soils for fasted and fed conditions. Derivation of the "average physiological state" correction factor. RIVM letter report 711701080, National Institute for Public Health and the Environment, Bilthoven, the Netherlands. Available via http://www.rivm.nl/bibliotheek/rapporten/711701080.pdf.

Kistemaker *et al.* (1998). Zo eet Nederland: resultaten van de Voedselconsumptiepeiling 1997-1998. Voedingscentrum, Den Haag.

Maddaloni M, Lolacono N, Manton W, Blum C, Drexler J, Graziano J (1998). Bioavailability of soilborne lead in adults, by stable isotope dilution. Environ. Health Perspect. 106 (Suppl 6): 1589-1594.

Minekus M, Marteau P, Havenaar R and Huis in 't Veld JHJ (1995). A multicompartimental dynamic computer-controlled model simulating the stomach and small intestine. ATLA 23: 197-209.

Minekus M, Smeets-Peeters M, Bernalier A, Marol-Bonnin S, Havenaar R, Marteau P, Alric M, Fonty G, Huis in't Veld JH (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Appl. Microbiol. Biotechnol. 53 (1): 108-114.

Oomen AG, Brandon EFA, Swartjes FA, Sips AJAM (2006). How can information on oral bioavailability improve human health risk assessment for lead-contaminated soils? Implementation and scientific basis. RIVM rapport 711701042, National Institute for Public Health and the Environment, Bilthoven, the Netherlands. Available via http://www.rivm.nl/bibliotheek/rapporten/711701042.pdf.

US EPA (2006). Standard Operating Procedure for an In Vitro Bioaccessibility Assay for Lead in Soil Available via http://www.epa.gov/superfund/health/contaminants/bioavailability/pdfs/pb_ivba_sop_final.pdf.

US EPA (2007) Estimation of relative bioavailability of lead in soil and soil-like materials using *in vivo* and *in vitro* methods. Office of Solid Waste and Emergency Response report nr. 9285.7-77.

Weis CP, Poppenga RH, Thacker BJ, Henningsen GM (1994). Design of pharmacokinetic and bioavailability studies of lead in an immature swine model. In: Lead in paint, soil, and dust: health risks, exposure studies, control measures, measurement methods, and quality assurance, ASTM STP 1226, M.E. Beard and S.A. Iske (Eds.). American Society for Testing and Materials, Philadelphia, PA, 19103-1187.

Appendix 1 US-EPA study

Calculation of relative bioavailability

The relative bioavailability (RBA) of the soil is equal to the absolute bioavailability (ABA, the fraction of lead that reaches the systemic circulation after oral ingestion) of the soil divided by the absolute bioavailability of lead acetate after oral ingestion. The method used to estimate the RBA of lead of a test soil, is based on the principle that equal absorbed doses of lead will produce equal biological responses. Below, this is explained in mathematical equations:

By definition,

$$Absorbed\ dose_{lead\ acetate} = given\ dose_{lead\ acetate} \times ABA_{lead\ acetate}$$

and

$$Absorbed\ dose_{test\ soil} = given\ dose_{test\ soil} \times ABA_{test\ soil}$$

When the responses (the concentrations in blood, liver, kidney and bone) are equal for lead acetate and the test soil, then the absorbed doses were equal and:

$$RBA = \frac{ABA_{test\ soil}}{ABA_{lead\ acetate}} = \frac{given\ dose_{lead\ acetate}}{given\ dose_{test\ soil}}$$

Relative bioavailability for 19 soils in USA

The relative bioavailability (RBA) of 19 test soils was thus estimated, by fitting mathematical models to the dose-response curves for each measurement endpoint (blood, liver, kidney and bone) and finding the ratio of doses (test soil/lead acetate) that gave equal responses. The final RBA for a test material was calculated as the (simple) average of the four endpoint-specific (blood, liver, kidney and bone) RBA values. The measured RBA values of the 19 soils varied from 1-105% (See Table 1).

Point estimate		
RBA	LB	UB
0.27	0.17	0.40
0.27	0.19	0.36
0.61	0.43	0.79
0.90	0.63	1.20
0.40	0.23	0.64
0.82	0.51	1.14
0.74	0.48	1.08
0.75	0.50	1.04
0.14	0.07	0.24
0.14	0.06	0.23
0.72	0.38	1.07
1.05	0.57	1.56
0.20	0.09	0.31
0.60	0.34	0.93
0.49	0.29	0.72
0.51	0.29	0.79
0.72	0.44	0.98
0.01	0.00	0.03
0.06	−0.01	0.15
0.86	0.43	1.52

Table 1   RBA values for 19 test soils from the US, calculated by averaging the results of the four endpoints blood, liver, kidney and bone. 5% lower confidence bound (LB) and 95% upper confidence bound are given (UB)

Appendix 2 Differences between the two study designs

Differences in study design between the Casteel assay and the alternative RIVM assay.

Parameter	Casteel assay	RIVM assay
Animals	Intact male pigs, 5-6 wks of age	Intact male pigs, 4-6 wks of age
No. of animals	4 per dose	6 per dose
Doses	3 dose levels	1 dose level
Dosing route	oral	Oral (test material, reference material), i.v. (reference material)
Dosing schedule	Twice daily, 2 hours before feeding	Twice daily, pigs in semi-fed state
Dosing duration	15 consecutive days	7 consecutive days
Observation period	Blood samples during dosing period (e.g., 0, 1, 2, 3, 4, 6, 9, 12, 15); tissues (femur, kidney, liver) collected on day 15	Daily blood samples during and following exposure, femur on day 7 post exposure
Endpoints measured	blood AUC (trapezoid rule) tissue lead concentration	blood AUC (PK model) femur lead concentration
ABA blood	not determined (typically)	(AUCRM,oral/D)/(AUCRM,iv/D)
ABA tissue	not determined (typically)	(femur lead concentration, oral/D)/femur lead concentration, iv/D)
RBA blood	simultaneous dose-AUC models for TM and RM solved for dose equivalence	(AUC TM,oral/D)/(AUC TM,oral/D)
RBA tissue	simultaneous dose-tissue concentration models for TM and RM, solved for dose equivalence	(CfemurTM/D)/(CfemurRM/D)

ABA = absolute bioavailability
RBA= relative bioavailability
TM = test material
RM= reference material
D = dose