Arsenic Metabolism by Human Gut Microbiota upon In Vitro Digestion of Contaminated Soils

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Competing interest declaration

The authors declare they have no competing financial interests.

List of abbreviations

iAs: inorganic arsenic, iAs^{V}: arsenate, iAs^{III}: arsenite, mTA: monothioarsenic acid; MMA^{V}: monomethylarsonic acid, MMA^{III}: monomethylarsonous acid, TMAO: trimethylarsine oxide; $MMMTA^{V}$: monomethylmonothioarsonic acid, DMA^{V}: dimethylarsinic acid, $DMMTA^{V}$: dimethylmonothioarsinic acid, $DMDTA^{V}$: dimethyldithioarsinic acid, IVG: *in vitro* gastrointestinal method; SHIME: Simulator of the Human Intestinal Microbial Ecosystem; L/S: liquid to soil ratio; HPLC-ICP-MS: high performance liquid chromatography-inductively coupled plasma mass spectrometry; ICP-OES: ICP-optical emission spectroscopy; HPLC-ESI-MS/MS: HPLC electrospray ionization tandem mass spectrometry

Abstract

Background: Speciation analysis is essential when evaluating risks from arsenic exposure. In an oral exposure scenario, the importance of presystemic metabolism by gut microorganisms has been evidenced with *in vivo* animal models and *in vitro* experiments with animal microbiota. However, it is unclear whether human microbiota display similar arsenic metabolism, especially when present in a contaminated matrix.

Objectives: Here, the metabolic potency of *in vitro* cultured human colon microbiota towards inorganic arsenic and arsenic contaminated soils was evaluated.

Methods: A colon microbial community was cultured in a dynamic model of the human gut. These colon microbiota were incubated with inorganic arsenic (iAs) and with arsenic contaminated urban soils. Arsenic speciation analysis was determined by high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS).

Results: A high degree of methylation was found both for colon digests of iAs (10 µg methylarsenical/g biomass/h) and of arsenic contaminated soils (up to 28 µg/g biomass/h). Besides the formation of monomethylarsonic acid (MMA^{V}), the highly toxic monomethylarsonous acid (MMA^{III}) was detected. Moreover, the finding of microbial thiolation leading to monomethylmonothioarsonic acid ($MMMTA^{V}$) was never described before. $MMMTA^{V}$, from which toxicokinetic properties are hardly known, was in many cases a major metabolite.

Conclusions: Presystemic arsenic metabolism is a significant process in the human body. Toxicokinetic studies aiming to completely elucidate the arsenic metabolic pathway would therefore benefit from incorporating the metabolic potency of human gut microbiota. This will result in more accurate risk characterization associated with arsenic exposures.

Introduction

Arsenic is a ubiquitous environmental contaminant presenting significant human health risks, as chronic exposure is associated with the development of cancer in the bladder, liver, kidney and lungs (Chen et al. 1992). Regions with a high geogenic arsenic background show an increased risk for elevated exposure by consumption of drinking water and diet. An additional exposure scenario in urban areas close to smelting and mining activities is the ingestion of contaminated soil and dust by children who display typical hand-to-mouth behavior. Although inorganic arsenic (iAs) may be the predominant form in contaminated soils, arsenic speciation changes during gastrointestinal transit are not well characterized. The gut represents a highly reducing environment and harbors a complex microbial community, which may contribute to the presystemic biotransformation of ingested arsenic (systemic metabolism being defined as all metabolic reactions carried out by human cells). Presystemic arsenic speciation analysis must therefore be considered an essential part of the risk evaluation process, especially with respect to toxicity, which is speciation dependent. In short, methylated trivalent species, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), and arsenous acid (iAs^{III}), are two orders of magnitude more cytotoxic than arsenic acid (iAs^{V}) (Naranmandura et al. 2007a). The methylated pentavalent species, monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}), present a tenfold lower toxicity than iAs^{V}, while trimethylarsine oxide (TMAO) is essentially non-toxic (Hirano et al. 2004).

In the human body, inorganic arsenic is sequentially methylated and predominately excreted as DMA^{V} in urine. This methylation process was originally considered a detoxification process, but the formation of reactive intermediates (MMA^{III} and DMA^{III}) has forced researchers to reconsider methylation as an activation process (Styblo et al. 2002). In addition, a recent study on human urine analysis following iAs exposure revealed new sulfur-containing methylated arsenic metabolites: monomethylmonothioarsonic acid ($MMMTA^{V}$) and

dimethylmonothioarsinic acid ($DMMTA^V$) (Naranmandura et al. 2007b; Raml et al. 2007), from which the mechanism of formation and toxicological profile are not yet fully characterized. Given the toxicological importance of arsenic speciation changes, it is clear that a complete risk characterization following arsenic exposure must include the possibility of presystemic metabolism by the microbial rich environment of the gastrointestinal tract.

The colon harbors a vast (10^{14} bacterial cells) and incredibly diverse (>1000 species) microbial community, which has the ability to metabolize xenobiotics far more extensively than any other part of the body (Sousa et al. 2008). Thus far, the presystemic biotransformation of arsenic was primarily studied with gut microbiota from animal models. The reduction of iAs^V to iAs^{III} by rat cecal bacteria was reported, as well as limited formation of MMA and DMA (Rowland and Davies 1981). Another study with rats, orally exposed to DMA^V, resulted in the detection of demethylated (iAs^V, MMA^V) and methylated (TMAO) urinary metabolites (Chen et al. 1995). Finally, the thiolation of methylated arsenic oxides (DMA^V, TMAO) in the cecal contents of a mouse (Kubachka et al. 2009a; Kubachka et al. 2009b) as well as the observed thiolation through *in vivo* experiments (Kuroda et al. 2004; Naranmandura et al. 2007b) was reported recently.

Presystemic arsenic metabolism in the human body has been less investigated (Hirner and Rettenmeier 2004). Nevertheless, it was reported that human gut microbes actively volatilize Bi and other metalloids, including As, through methylation and hydrogenation (Michalke et al. 2008). Moreover, Meyer et al. postulated that gut methanogens play a crucial role in metalloid volatilization, thereby exerting toxic effects to the human body – not only by direct interaction with the host, but also by disturbing the endogenous gut microbiota composition and metabolism (Meyer et al. 2008). Finally, a thorough *in vitro* exploration with the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), a dynamic human gastrointestinal simulator revealed a high microbial metabolic potency towards metal(loid)s (Diaz-Bone and

Van de Wiele 2009). This was demonstrated by the finding of significant volatilization of As, Se, Bi, Te and Sb, the formation of highly toxic AsH_3 (arsine) and $(CH_3)_2Te$ and the discovery of 2 new arsenic-sulfur metabolites.

These data indicate the need for more studies with human gut microorganisms, which can confirm the presystemic metabolism as observed with animal gut microbiota. This study will therefore investigate the metabolic potency of human gut microorganisms towards iAs and arsenic from contaminated urban soils. It allows making an assessment of i) the importance of presystemic arsenic biotransformation upon an oral exposure scenario and ii) the actual speciation of arsenic that enters the bloodstream upon gastrointestinal digestion.

Experimental section

Chemicals and media.

Degassed, ultrapure 18 mΩ water (DDI; Millipore, Bedford, MA, USA) was used for the preparation of the chromatographic mobile phase as well as standard stock solutions. ACS grade ammonium nitrate, ammonium dihydrogen phosphate (Fisher Scientific, Pittsburgh, PA, USA) and technical grade ethylenediamine tetracetic acid, tetrasodium salt dehydrate (Fisher Scientific, Fair Lawn, NJ, USA) were utilized in the chromatographic mobile phase. Stock solutions of inorganic arsenic (As^{III} and As^{V}) were obtained from Spex Industries (Metuchen, NJ, USA). Certified stock solutions of monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}) were obtained from Chem Service (West Chester, PA). Professor William R. Cullen (Department of Chemistry, University of British Columbia, Vancouver, BC, Canada) provided tetramethyl-cyclo-tetraarsaoxane [cyclo-$(CH_3AsO)_4$] crystals that were synthesized and characterized as described elsewhere. These crystals were stored at -21°C and were hydrolyzed by degassed, deionized water at the time of analysis to obtain a stock solution of a MMA^{III} and MMA^{V} mixture (Cullen et al. 1989). Sodium arsenate ($Na_2HAsO_4.7H_2O$), methionine, methylcobalamine, and glutathion were obtained from Sigma-Aldrich (St.-Louis, MO, USA). Arsenate stock solutions were prepared in deionized water at 4500 mg As/L and 45 mg As/L.

Soils.

US EPA kindly provided four As-contaminated soils, which originated from urban areas around former smelting sites. All soils were sieved at 250 µm prior *in vitro* gastrointestinal incubation. This reflects the size of particles that most likely sticks to the hands of exposed humans (Kelly et al. 2002). Soil specifications are reported in Table 1.

Production and characterization of colon microbiota for SHIME

The *in vitro* colon microbial community used in this study was cultured and maintained in a modified Simulator of the Human Intestinal Microbial Ecosystem (SHIME), which consisted of 4 compartments simulating the stomach, small intestine and both the proximal and distal colon. A detailed description of the SHIME, the carbohydrate-based medium and the *in vitro* colon microbiota can be consulted in (Van de Wiele et al. 2004). Briefly, an in-house culture developed from fecal microbiota previously obtained from a 29-year old male volunteer (who had no history of antibiotic treatment in the 6 months prior to the study) were inoculated in the different colon compartments. The carbohydrate-based medium was fed 3 times/d to the SHIME reactor and thus provided digested nutrition for the colon microbiota. After 3 weeks of adaptation, a stable microbial community was obtained in the respective colon compartments. The distal colon microbial fermentation activity (short chain fatty acid production and ammonium production) and community composition was investigated and found to be consistent with that of previous SHIME runs and the *in vivo* situation (Molly et al. 1994; Van de Wiele et al. 2004) (Supplementary Table 1).

Non-continuous incubation studies.

Metabolic potency of fecal microbial inoculum.

The first experiment constituted a screening phase to test whether the fecal microbial community from human origin actively metabolized arsenic. The microbial community was isolated from a fecal sample as previously described (Molly et al. 1994). Thirty mL of microbial fecal suspension was sampled and placed in 60 mL serum bottles and incubated with $NaH_2AsO_4.7H_2O$ (iAs^V) at a concentration of 90 mg iAs^V/L, this in analogy with (Herbel et al. 2002). Serum bottles were capped with butyl rubber stoppers, impervious to O_2, and subsequently made anaerobic by flushing with N_2 gas for 30 minutes. Samples were incubated at 37°C on a rotary shaker (150 rpm) for 48 h. The effect of specific methyl group donors towards microbial As

methylation was evaluated by comparing methionine (5 mmol/L) and methylcobalamin (5 mmol/L) amended samples with control samples. The effect of glutathione as a reducing agent was evaluated by comparing glutathione amended samples (10 mmol/L) with control samples. Duplicate incubations were performed on two different days to evaluate the reproducibility. The control for these experiments involved incubating the sample in the presence of heat-sterilized fecal microbiota. A scheme of the experimental setup is represented in Supplemental Figure 1.

Metabolic potency of colon microbiota towards As from contaminated soils.

The objective of the second experiment was to screen for microbial speciation changes of iAs^{V} at more relevant concentrations (i.e. 50-500 µg/L range) by mimicking conditions of oral exposure to environmental samples. In addition, four As-contaminated soil samples (1 slag soil and three from urban sites) were subjected to a gastrointestinal digestion procedure. To better mimic *in vivo* conditions, all gastrointestinal stages – gastric, small intestine and colon – were simulated. We combined the *in vitro* gastrointestinal method (IVG) from Ohio State University with the SHIME to subsequently simulate stomach and small intestine (IVG) and colon (SHIME) conditions, respectively. The IVG-method was previously validated against *in vivo* data for arsenic bioaccessibility (Rodriguez and Basta 1999) whereas the SHIME has been validated against *in vivo* data for microbial community composition and metabolic activity towards drugs and phytoestrogens (Molly et al. 1994; Possemiers et al. 2006).

Soils were incubated in the gastric and intestine solution (30 mL) of the IVG protocol at a liquid to soil (L/S) ratio of 150 (Rodriguez and Basta 1999). These intestinal digests from the IVG protocol were subjected to colon conditions by adding 30 mL of the colon suspension sampled from the distal colon compartment of the SHIME reactor, resulting in a L/S ratio of 300 for the soil digests. The vessels containing the colon digests were capped with butyl rubber stoppers and

subsequently flushed with N_2 for 30 minutes to obtain anaerobic conditions and incubated on a shaker at 150 rpm at 37°C for 18 h. A schematic representation of the experimental setup can be consulted in supplemental figure 1.

Sample treatment.

To preserve the speciation of As in the colon digests, all samples were flash frozen with liquid nitrogen upon incubation and subsequently stored at -80°C. Prior to HPLC-ICP-MS analysis, samples were thawed and diluted appropriately with 20 mmol/L $(NH_4)_2CO_3$ at pH 9.0 to minimize sulfur-oxygen exchange while awaiting analysis (Conklin et al. 2008). Upon complete thawing, the sample was vortexed and centrifuged for 10 minutes at 10400 rcf with an Eppendorf 5810R (Brinkman Instruments, Westburg, NY, USA) to separate soluble As species from soil-bound As. The supernatant was filtered through a Millex-LCR 0.45µm filter (Millipore, Bedford, MA, USA) with a Luer-Lok 10mL syringe (BD, Franklin Lakes, NJ, USA). Finally, filtrates were diluted with the mobile phase and injected on HPLC. The sum of the arsenic species in the filtrate observed chromatographically, was considered the bioaccessible fraction. Total arsenic measurements on the digest filtrates were performed with inductively coupled plasma optical emission spectroscopy (ICP-OES). This allowed calculating the chromatographic recovery, which quantifies to what extent the sum of the chromatographic As species comprises the total amount of arsenic in the digest filtrates.

As speciation analysis by HPLC-ICP-MS.

Sample supernatants were analyzed with HPLC (Agilent 1100)-ICP-MS (Agilent 7500ce) for arsenic specific detection at *m/z* 75. Separation of As-oxides was performed on a PRP-X100 HPLC column (250 mm x 4.1 mm, 5 µm). The mobile phase was a solution of NH_4NO_3 (10 mmol/L), $NH_4H_2PO_4$ (10 mmol/L) and EDTA (500 mg/L) at pH 4.57 in distilled water (Separation 1). The flow rate was 1.0 mL/min

while the sample injection volume was 100 µL. The retention times of the separated compounds were: As^{III} 3.6 min, DMA^{V} 4.2 min, MMA^{III} 5.5 min, MMA^{V} 7.1 min, and As^{V} 8.9 min which are similar as previously reported (Yathavakilla et al. 2008). This separation was used for the quantification of the arsenic species of interest. Arsenic sulfides were identified by retention time matching between samples and fortified samples. Using chromatographic separation 1, monothioarsonic acid eluted at 15 min, while $MMMTA^{V}$ eluted at 18.6 min. The synthesis, the chromatographic confirmation of these arsenic sulfides and sample analysis can be consulted in supplemental information.

Results.

In the first experiment, we assessed the metabolic potency of the human fecal microbial inoculum towards high levels of iAs^{V} (90 mg/L). iAs^{V} was efficiently (more than 94%) reduced to iAs^{III} following the 48 h incubation with both active and sterilized fecal microbiota (Table 2), probably due to the highly reducing conditions (redox potential was -180 mV). Incubation with sterilized fecal microbiota did not lead to thiolated or methylated arsenicals. In contrast, incubation of iAs^{V} with active fecal microbiota resulted in the production of monothioarsonic acid (mTA) in non-amended (2.2 ± 3.1 mg/L) and methionine (0.8 ± 1.1 mg/L) amended samples. Interestingly, methylation was only observed in the presence of methylcobalamin. Addition of the latter displayed a significant methylation of iAs (18%) with MMA^{V} (13.0 ± 1.4 mg/L) being more dominant than MMA^{III} (2.6 ± 1.4 mg/L). The addition of both methylcobalamin and glutathione as a reducing agent increased the methylation to 28% with MMA^{III} (10.5 ± 5.4 mg/L) becoming equally important as MMA^{V} (11.3 ± 5.6 mg/L).

These preliminary data convinced us that the selected microbial community had the potency to actively metabolize iAs^{V}. The SHIME reactor was therefore inoculated with this fecal microbial inoculum and after three weeks of adaptation, a stable microbial community was obtained in the proximal and distal colon compartments. We regularly sampled the distal colon compartment to perform colon incubations on iAs^{V} and As-contaminated soil samples that had already gone through a gastric and intestinal digestion. Characterization of the colon digests consisted of determining arsenic bioaccessibility and arsenic speciation. As the bioaccessibility determination was based upon the sum of all chromatographically detected (LC-ICP-MS) As species in the filtrates (0.45µm) of the colon digests, the chromatographic recovery was first calculated. The sum of the concentrations of chromatographically detected As species in the colon filtrates was divided by the total As concentration in the colon filtrates, as measured by ICP-OES. The chromatographic recoveries for all colon

digests, except for that of soil 4, were satisfactorily high: 93 ± 7% on average (the recovery of the soil 4 digest excluded) (Supplementary Table 2). Hence, the majority of As species present in these digest supernatants could be detected with the HPLC-ICP-MS protocol. Bioaccessibility calculations for these digests displayed the highest value (75.5%) for the iAs^{V} incubated colon digest, while colon incubation of the contaminated soils resulted in arsenic bioaccessibility values of 24% (soil 1), 44% (soil 2) and 36% (soil 3) (Table 3). In sharp contrast, the As bioaccessibility in the colon digest of soil 4 was only 0.3%. Even when taking into account the low chromatographic recovery of 15%, a low As bioaccessibility of 2.4% was obtained, which is still an order of magnitude lower than the bioaccessibility values for the other soil digests. Overall, colon bioaccessibility values (Table 3) for the 4 soils were consistently lower than the corresponding intestinal bioaccessibility values (Table 1) as obtained with the IVG-method.

The most important part of this study consisted of the As speciation analysis of the colon digests following the gastrointestinal incubation of iAs^{V} and the four contaminated soils. The original analytical protocol was optimized to detect the presence of iAs^{III}, iAs^{V}, MMA^{III} and MMA^{V}. We detected an additional arsenic species, monomethylmonothioarsonic acid ($MMMTA^{V}$), in many of the colon digests. $MMMTA^{V}$ was initially identified using a combination of retention time matching and by fortifying the sample with the suspected standard using Separation 1 with ICP-MS detection. A second chromatography, Separation 2 (see supplemental information), was used for ICP-MS and ESI-MS detection, because the mobile phase of Separation 1 was not compatible with ESI-MS detection. Figure 1 shows HPLC-ICP-MS mass chromatograms of *m/z* 75 (^{75}As) and HPLC-ESI-MS mass chromatograms of *m/z* 155 ($[M-H]^-$) for a $MMMTA^{V}$ standard and a SHIME extract using Separation 2. The retention times of the $MMMTA^{V}$ in the standard and $MMMTA^{V}$ in the sample were slightly offset as the matrix of the soil extract caused the decreased retention of $MMMTA^{V}$ on the C_{18} column. MS/MS of *m/z* 155 yielded a product ion of *m/z* 137

(loss of H_2O) and, to a lesser extent, a product ion of *m/z* 121 (due to $CH_2AsO_2^-$) and *m/z* 140 (loss of CH_3). The molecular mass of 155 and corresponding fragments were consistent with other reports for $MMMTA^V$ (Yathavakilla et al. 2008).

Significant arsenic methylation was detected upon colon incubation of 225 µg iAs^V/L (Figure 2). The sum of the concentrations of MMA^V (31.0 µg/L), MMA^{III} (4.5 µg/L) and $MMMTA^V$ (43.7 µg/L) exceeded that of iAs^V (39.0 µg/L) and iAs^{III} (34.8 µg/L)). In contrast, the iAs species were predominantly present in colon digests of soils 1, 2 and 3, while they were the only arsenic species in the colon digest of slag soil 4 (Figure 2). The colon digest of soil 1 displayed a methylation percentage of 4.7% with MMA^V (17 µg/L) and $MMMTA^V$ (23 µg/L) as detected methylarsenicals. The methylation percentage for colon digests of soil 2 (22.8%) and soil 3 (21.2%) was higher with soil 2 displaying MMA^V (111 µg/L), MMA^{III} (9 µg/L) and $MMMTA^V$ (158 µg/L) and soil 3 only displaying MMA^V (28 µg/L) and $MMMTA^V$ (68 µg/L). Finally, no methylated As species were detected in the colon digests of slag soil 4.

Summarizing the *in vitro* As speciation changes by human gut microorganisms, we calculated the specific production rates of methylated arsenicals by taking into account the initial microbial biomass and arsenic concentrations. A methylation rate of 10 µg methylarsenicals per g biomass per hour was obtained for the colon digest of iAs^V (Table 3). Although no methylarsenicals were detected in colon digests of the slag soil 4, the presence of the other soil matrices did not necessarily lower the above-mentioned methylation rate. Methylation rates of 4, 29 and 10 µg/g/h were obtained for colon digests of soil 1, 2 and 3, respectively (Table 3).

Discussion.

This study demonstrates that human colon microorganisms have the potency to actively metabolize arsenic into methylated arsenicals and thioarsenicals, which indicates that presystemic arsenic metabolism may not be neglected when assessing risks from oral arsenic exposure. This was observed both upon colon incubation of iAs and As contaminated soils. These findings parallel those from studies with animal gut microbiota (Hall et al. 1997; Rowland and Davies 1981) and suggest the existence of a presystemic arsenic metabolism in the human body. The most important result was the detection of significant levels of $MMMTA^{V}$ in colon digests of both iAs^{V} (25% of the bioaccessible As) and of As contaminated soils (up to 20% of the bioaccessible As). $MMMTA^{V}$ production from iAs^{V} by human colon microbiota has, as far as we know, never been described before. It resembles the methylation and thiolation of DMA^{V} into trimethylarsine sulfide by mouse cecal microbiota (Kubachka et al. 2009a) and the production of methylated thioarsenicals starting from DMA^{V} with rat intestinal microbiota (Kuroda et al. 2004; Yoshida et al. 2001). Yet, mammalian cells also have the ability to form methylated thioarsenicals. Dimethylmonothioarsinic acid ($DMMTA^{V}$) and dimethyldithioarsinic acid ($DMDTA^{V}$) were rapidly (5 minutes) detected upon injection of DMA^{III} in rats (Kuroda et al. 2004) and DMA^{III} was converted to $DMDTA^{V}$ by human red blood cells (Naranmandura and Suzuki 2008).

The finding of presystemic $MMMTA^{V}$ formation by human gut microorganisms raises questions over its toxicological importance. Although the absorption kinetics of $MMMTA^{V}$ and other thiolated arsenicals across the epithelium are unknown, there is evidence that some methylated thioarsenicals elicit a higher toxicity than iAs^{V} due to their more efficient absorption by mammalian cells (Naranmandura et al. 2007a). Preliminary cytotoxicity (Naranmandura et al. 2007a; Yoshida et al. 2003) and genotoxicity (Kuroda et al. 2004) data for $DMMTA^{V}$ show similar levels of toxicity as for trivalent arsenic species. The observations in this study emphasize the need for

investigating the behavior of $MMMTA^{V}$ in the gut lumen and the absorption rate across the intestinal epithelium. In addition, the mechanism behind the microbial production pathway needs to be elucidated. Interestingly, $MMMTA^{V}$ levels in the colon digests correlated with those of MMA^{V} (R^2=0.76), whereas the correlation with levels of MMA^{III} was much lower (R^2=0.42). This seems to indicate that $MMMTA^{V}$ in the colon digests arises from the thiolation of MMA^{V}, which would correspond with earlier observations describing the interconversion between oxide and sulfide forms of MMA^{V}, DMA^{V} and TMAO (Conklin et al. 2008). The sulfide source may originate from microbial sulfate reduction to H_2S, which is a common process in the colon environment (Deplancke et al. 2000) and can trigger the formation of thioarsenosugars upon the incubation of arsenosugars with mouse cecal contents (Conklin et al. 2006). The role of sulfate reducing microorganisms in the presystemic production pathway of $MMMTA^{V}$ must therefore be further studied.

The significant formation of MMA^{V} and MMA^{III} following incubation of iAs^{V} with colon microorganisms was not unexpected. Arsenic methylation by rodent gut microbes (Hall et al. 1997; Rowland and Davies 1981) and human gut microbes (Diaz-Bone and Van de Wiele 2009; Meyer et al. 2008) was described previously. Taking into account the initial biomass concentration, we observed specific methylation rates of 10 µg methylarsenicals per hour per gram of biomass. This roughly corresponds to 130 pmol/h/mg biomass, which is higher than 16 pmol/h/mg obtained with rat cecal microbiota (Hall et al. 1997). Interestingly, the presence of a soil matrix did not necessarily result in lower arsenic methylation rates. Yet, soil-dependent parameters may have affected the methylation rate. Firstly, comparison of the mineralogy from soils 1, 2 and 3 with that of slag soil 4 showed an important difference in reactive Fe-oxide content (Table 1), which is highly efficient in sorbing arsenic (Beak et al. 2006). The reactive Fe-oxide content in slag soil 4 was particularly high (18759 µg/kg, Table 1), presumably leading to a much lower arsenic availability to colon microorganisms (0.3% bioaccessibility) and thus also limiting

methylation. This observation may confirm earlier observations of slag soil mineralogy significantly decreasing arsenic bioavailability (Davis et al. 1996). A second element in the soil-dependent arsenic methylation may be the difference in toxic elements. In comparison with the first three soils, slag soil 4 contained high amounts of Cd, Cr, Cu, Mo, Pb, and Zn (Table 1), which may present toxicity towards intestinal microorganisms. Our finding of a 70% lower fermentation activity in soil 4 colon digests versus colon digests of the other soils (data not shown) may support the assumption of slag soil induced toxicity. The actual relationship between gut microbial arsenic metabolism and soil characteristics therefore needs further study.

A final aspect of this study concerns the metabolic potency of fecal microbes towards high levels of iAs^{V} (90 mg iAs^{V}/L) and the influence of cofactors. Firstly, non-amended colon digests of iAs^{V} resulted in the efficient reduction to iAs^{III} and the production of mTA. Similar to the finding of $MMMTA^{V}$, the formation of mTA may result from an oxygen-for-sulfur exchange in iAs^{V}, due to the availability of sulfide, originating from the above-mentioned microbial sulfate reduction. The absence of mTA in glutathione-amended samples may be explained by the complete reduction of iAs^{V} into iAs^{III} by glutathione as reducing agent. The effect of methyl group donors was also evaluated. In contrast to methionine, methylcobalamin may be an effective methyl group donor, resulting in the efficient methylation (19%) of iAs^{V} into MMA^{V} and MMA^{III} (Table 2). The methylation efficiency was increased to 25% upon co-supplementation of methylcobalamin and glutathione. This was attributed to the increased reduction of MMA^{V} into MMA^{III} by glutathione as reducing agent. In contrast to the colon digests with low levels of iAs^{V} (225 µg/L), no $MMMTA^{V}$ was detected in the fecal digests. A probable explanation is the difference in experimental setup, the difference in microbial community composition and activity or a difference in sulfide availability. These observations confirm a previous report stating that the addition of cofactors may increase arsenic methylation by enteric microorganisms,

yet it is not a prerequisite for the methylation of low levels of As (µg/L range) (Hall et al. 1997).

This study provides evidence for the existence of significant presystemic arsenic metabolism by human gut microorganisms, however the relevance for the total risk of oral arsenic exposure is not yet clear. So far, the *in vitro* approach for assessing the risks from oral contaminant exposure mainly involved the use of models that focus on gastric and intestine processes. Methylation of arsenic by intestinal microorganisms was thought to contribute little to the overall methylation *in vivo* (Vahter and Gustafsson 1980) because iAs^{V} and iAs^{III} are rapidly absorbed in the small intestine (Vahter 1983), especially when arsenic is ingested in a soluble matrix, e.g. drinking water. However, soil-bound and/or dietary-bound arsenic may follow a different digestion scenario in the gut and a large fraction may end up in the colon lumen where it is subjected to the resident microbial community. The finding of $MMMTA^{V}$ and the highly toxic MMA^{III} as metabolites from human colon microorganisms indicates that presystemic methylation will not lead to detoxification. In addition, *in vitro* studies with Caco-2 cells suggest that the absorption of methylated arsenicals (DMA^{V}: 10.0%, TMAO: 10.9%) is more efficient than that of iAs^{III} (5.8%) and iAs^{V} (1.6%) (Laparra et al. 2005, 2007). Information on intestinal absorption of methylated thioarsenicals should be acquired in future research.

A final remark considers the variability between individuals regarding presystemic arsenic metabolism. We only investigated the gut microbiota from one human individual. However, the interindividual variability in the gut microbial community composition is very high. Hence, we expect gut microbiota from different individuals to display distinct arsenic metabolic profiles. Such interindividual variation in the metabolism by human gut microbiota was previously reported for ingested phytoestrogens (Bolca et al. 2007), but interestingly also for the metalloid bismuth (Michalke et al. 2008). Therefore, variability in gut microbial arsenic metabolism

should be given equal attention as the genetic variations that may govern interindividual differences in arsenic response (Hernandez and Marcos 2008).

Conclusion

This study shows that presystemic metabolism of soil-derived arsenic may be relevant in the human body when significant amounts of arsenic become available to colon microorganisms. The absorption kinetics of methylated arsenicals and thioarsenicals across the gut epithelium and their toxicity need further elucidation. We propose to incorporate the metabolic activity of human colon microorganisms when developing new toxicokinetic models that assess risks from oral arsenic exposure. Mikov nicely summarized the importance of gut microbiota stating that gut microbial metabolism must be considered an integral part of drug/xenobiotic metabolism and toxicity studies (Mikov 1994). In this context, knowledge about gut microbial metabolism must be translated to metal(loid) biotransformation as well.

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Tables and Figures

Table 1. Characteristics and elemental composition of the four arsenic contaminated soils

Characteristics	Soil 1	Soil 2	Soil 3	Soil 4 (=slag)
organic C (%)	5.7	4.0	3.1	2.2
pH[a]	6.1	6.3	5.0	7.2
Electric conductivity (dS/m)	0.3	0.5	0.9	0.5
Fe (mg/kg)	14800	15250	12100	202000
Mn (mg/kg)	429	525	207	1640
Reactive Fe[b]	3489	3592	1634	18759
% Bioaccessibility with IVG-method [c]	58.5	62.4	47.7	1.0
Toxic Trace Element Content (mg/kg)				
As	990	829	379	837
Cd	9.7	5.5	1.5	28.9
Cr	32.0	31.4	28.7	187
Cu	51.9	60.1	22.2	1520
Mo	2.1	2.4	1.5	73.9
Ni	12.5	10.9	9.3	10.4
Pb	885	602	172	8702
Se	330	430	127	294
Zn	562	803	151	12500

[a] soil pH was determined in a 1:10 (wt/vol) aqueous soil slurry

[b] Fe dissolved by acid ammonium oxalate extraction

[c] (Whitacre 2009)

Table 2. Influence of co-factors towards microbial metabolism during *in vitro* fecal incubation of iAs^{V} at 90 mg/L. Values are represented as averages (mg/L) from duplicate incubation experiments ± standard deviation.

	As^{V}	As^{III}	MMA^{V}	MMA^{III}	mTA	Recovery (%)
Active microbiota						
No co-factor	1.7 ± 2.3	97.6 ± 2.5	ND[a]	ND	2.2 ± 3.1	113
Methylcobalamin (Me-B12)	2.6	65.3	13.0 ± 1.4	2.6 ± 1.4	ND	93
Methionine (Meth)	1.7 ± 2.3	103.4 ± 3.1	ND	ND	0.8 ± 1.1	118
Glutathion (GSH)	ND	93.2 ± 18.8	ND	ND	ND	104
Me-B12 / Meth / GSH	2.0 ± 1.2	53.6 ± 9.7	11.3 ± 5.6	10.5 ± 5.4	ND	86
Sterilized microbiota						
No co-factor	5.6[b]	87.6	ND	ND	ND	104
Methylcobalamin (Me-B12)	4.6	72.7	ND	ND	ND	86
Methionine (Meth)	4.6	86.8	ND	ND	ND	102
Glutathion (GSH)	ND	84.6	ND	ND	ND	94
Me-B12 / Meth / GSH	3.5	83.7	ND	ND	ND	97

[a] ND = not detected

[b] Incubation tests with sterilized microbiota were performed once, hence no standard deviation is available

Table 3. Percentage bioaccessibility of arsenic and biomass specific production rate of methylated arsenicals by colon microorganisms following *in vitro* colon digestion of iAs^V (225 µg/L) and As contaminated soils. Values are represented as averages (n=3) ± standard deviation.

	% Bioaccessibility [a]	Methylation rate [b] (µg/h/g biomass)
$Na_2HAsO_4.7H_2O$	76 ± 14.3	10.0 ± 4.0
Soil 1	24 ± 4.3	10.0 ± 1.8
Soil 2	44 ± 3.9	4.2 ± 1.6
Soil 3	36 ± 1.5	28.9 ± 3.4
Soil 4	0.3 ± 0.1	ND[c]

[a] The percentage bioaccessibility was calculated by dividing the sum of detected arsenic species (iAs^V, iAs^{III}, mTA, MMA^V, MMA^{III} and $MMMTA^V$) in the colon digest filtrate by the total amount of arsenic that was incubated under colon conditions.

[b] The biomass specific methylation rate was calculated by dividing the sum of methylated arsenicals (MMA^V, MMA^{III} and $MMMTA^V$) in the colon digest filtrate by the initial biomass concentration and the incubation time.

[c] ND = not detected

Figure 1. Identification of monomethylmonothioarsonic acid ($MMMTA^{V}$) by HPLC-ICP-MS and HPLC-ESI-MS/MS (using *Separation 2* in supplemental information). The grey and black traces in chromatograms represent analysis of a soil extract and $MMMTA^{V}$ standard, respectively. The MS/MS spectra of *m/z* 155 are inlayed within each MS spectra.

Figure 2. Concentration of chromatographically detected arsenic species (iAs^{V}, iAs^{III}, MMA^{V}, MMA^{III} and $MMMTA^{V}$) in colon digests of iAs^{V} (225 µg/L) and of As contaminated soils. Values are represented as averages (n=3) ± standard deviation. Note that different scales apply to the arsenic concentration (Y-axis) between colon digests of iAs^{V} and contaminated soils.