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ORAL EXPOSURE TO ENVIRONMENTAL CONTAMINANTS: PROCESSES OF BIOAVAILABILITY AND INTERACTIONS WITH INTESTINAL MICROORGANISMS

ORALE BLOOTSTELLING AAN MILIEUCONTAMINANTEN: PROCESSEN VAN BIOBESCHIKBAARHEID EN INTERACTIES MET INTESTINALE MICRO-ORGANISMEN

door

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Opgedragen aan mijn vader
Luc Van de Wiele
1949-1987

Notation index

ADI	Acceptable Daily Intake
AhR	aryl hydrocarbon receptor
ALARA	as low as reasonably achievable
ANOVA	analysis of variance
Arnt	aryl hydrocabon receptor nuclear translocator
AT	period over which exposure will be averaged
BA	absolute bioavailability
BTEX	benzene, toluene, ethylbenzene, xylene
BW	body weight
CFU	colony forming units
CMC	critical micelle concentration
C_s	chemical concentration in the soil at the point of contact
CYP	cytochrome P450 enzyme complex
D_{abs}	absorbed dose
D_{adm}	administered dose
DAD	diode array detection
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRE	DNA responsive elements
DW	dry weight
EC_{50}	50% effect concentration
ED	exposure duration
EDTA	ethylenediaminetetraacetic acid
EE2	17β-ethynyl-estradiol
EF	exposure frequency
ER	estrogen receptor
ERA	environmental risk assessment
ESI	electrospray ionization
F	bioavailable fraction
F_{Abs}	absorbed fraction
F_{BAcc}	bioaccessible fraction
F_{Met}	fraction that passes liver without metabolization
F_s	fraction absorbed from soil under environmental conditions

F_{sm}	fraction absorbed from the study matrix in the toxicity study
FISH	fluorescent in situ hybridization
FOS	fructooligosaccharides
GC	gas chromatography
GI	gastrointestinal
HAA	heterocyclic aromatic amine
HDPE	high density polyethylene
HHRA	human health risk assessment
HPLC	high-performance liquid chromatography
ICP-AES	inductively Coupled Plasma-Atomic Emission Spectrometry
ICP-MS	inductively Coupled Plasma-Mass Spectrometry
IQ	2-amino-3-methylimidazo[4,5-f] quinoline
IR	incidental ingestion rate of soil
kDa	kilodalton
K_{ow}	octanol/water partition coefficient
L/S ratio	liquid (mL) to soil (g) ratio
LC-MS	liquid chromatography - mass spectrometry
LD_{50}	50% lethal dosis
LOD	limit of detection
LOQ	limit of quantification
MDR	multiple drug resistance
MPL	maximum permissible level
MQ	milli-Q® water
mRNA	messenger ribonucleic acid
MS	mass spectrometry
m/z	mass to charge ratio
NOEL	no observable effect level
NEL	no effect level
NL	negligible level
PAH	polycyclic aromatic hydrocarbons
PBET	Physiologically Based Extraction Test
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-dioxin
PCDF	polychlorinated dibenzo-furanon
PCR	polymerase chain reaction
PEC	predicted environmental concentration
PM	particulate material

PNEC	predicted no effect concentration
PNEL	predicted no effect level
POP	persistent organic pollutants
RAF	relative absorption factor
RBA	relative bioavailibility
RNA	ribonucleic acid
RSD	relative standard deviation
SBET	Simple Bioaccessibility Extraction Test
SCFA	short chain fatty acids
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
SPE	solid phase extraction
TCDD	tetrachloro dibenzo-dioxine
TIM	TNO intestinal model
TLV	Threshold Limit Values

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CHAPTER 1

Literature review

1. Environmental contamination and human health

1.1 Introduction

"De aere, aquis et locis" from Hippocrates (460-377 BC) is the earliest human ecology study which recognized the relationship between environmental pollution and human health and which reported on the influence of nutrition, occupation and climate in causing diseases. Likewise, Ancient Roman writers such as Vitruvius, Plutarch and Pliny the Elder reported the toxic effects of mercury, lead and zinc towards workers in industrial sites such as mines and smelters (Vitruvius, 25BC). Recent reports suggest that about one quarter to one third of the human diseases in the world can be directly attributed to environmental factors (EEA, 2003). Therefore, a lot of attention has been payed by international institutions such as the World Health Organization (WHO), the Organization of Econonomic Cooperation and Development (OECD), Environmental Protection Agency (EPA) and the European Centre For Ecotoxicology and Toxicology of Chemicals (ECETOC) since the 1990s to the development of Human Health Risk Assessment (HHRA) and Environmental Risk Assessment (ERA) studies. Although the cause-effect relationship from pure chemicals is reasonably understood, the actual health consequences from these compounds when present in environmental matrices or mixed with other contaminants, are difficult to assess. Additionally, the complexity of environmental pollution phenomena is ever increasing, showing the importance and relevance to investigate, clarify and identify the relationship between environmental pollution and human or environmental health. The complexity of the exposure pathways of environmental contaminants towards human or ecological receptors can be observed from the multitude of processes that determine the environmental fate and concentrations of contaminants in the four main environmental compartments: air, water, soil and biomass (Figure 1.1).

A domain of risk assessment which needs further attention is the human exposure to soil-bound contaminants by ingestion. A first issue is that chemicals may have different degrees of binding to soil, nutrition or liquid matrices, thus changing their availability for uptake by the human body. This results in different degrees of internal exposure to the chemical, depending on the compound, the matrix or other environmental factors. Those processes that precede this biological availability or in

short, bioavailability, are currently not well understood. Scientific research will allow to develop better methods by which a more accurate prediction of the bioavailable fraction of ingested contaminants may be obtained.

Secondly, there is increasing concern about the behavior of ingested contaminants in the gastrointestinal tract with respect to their biological activity. Although the bioactivation or inactivation of chemicals by human biotransformation enzymes is extensively studied and well understood by environmental scientists, pharmacologists and toxicologists, there is only limited knowledge on the biotransformation potency from the vast microbial community residing in the human large intestine. It is believed that the microbial metabolic potency may be as diverse as the human biotransformation processes. This may have considerable consequences when assessing the health risks from ingested contaminants, since the toxicity of bioavailable chemicals largely depends on their degree of metabolization.

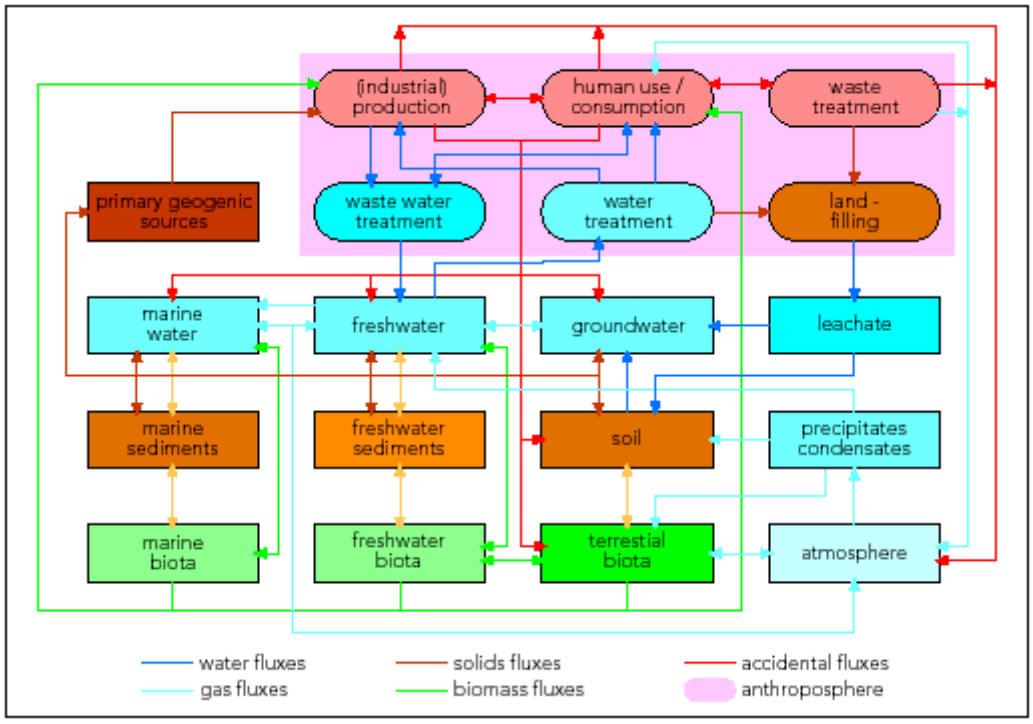

Figure 1.1 Important pathways for the transfer of chemical substances between several compartments of the natural and man-made environment. (Source: EEA, 1999)

1.2 Human health risk assessment

1.2.1 Risk management process

The 1990s have brought considerable activity in the field of risk assessment. With regards to environmental pollution, regulatory decisions that are issued by international and national environmental institutes, are based upon risk assessment studies. The objective of human health risk assessment is to determine the likelihood that exposure to a toxic chemical will have adverse effects on human health and to quantitatively estimate the magnitude of the effects. Human health risk assessment is not only a central theme in the management of environmental pollution hazards, but also in the control of chemicals that are brought on the market. In essence, the process of managing health risks consists of the risk assessment step and the risk management step. The assessment of risks towards human health can basically be split up in four major components (Figure 1.2) (NRC, 2003): hazard identification, exposure assessment, dose-response assessment and risk characterization.

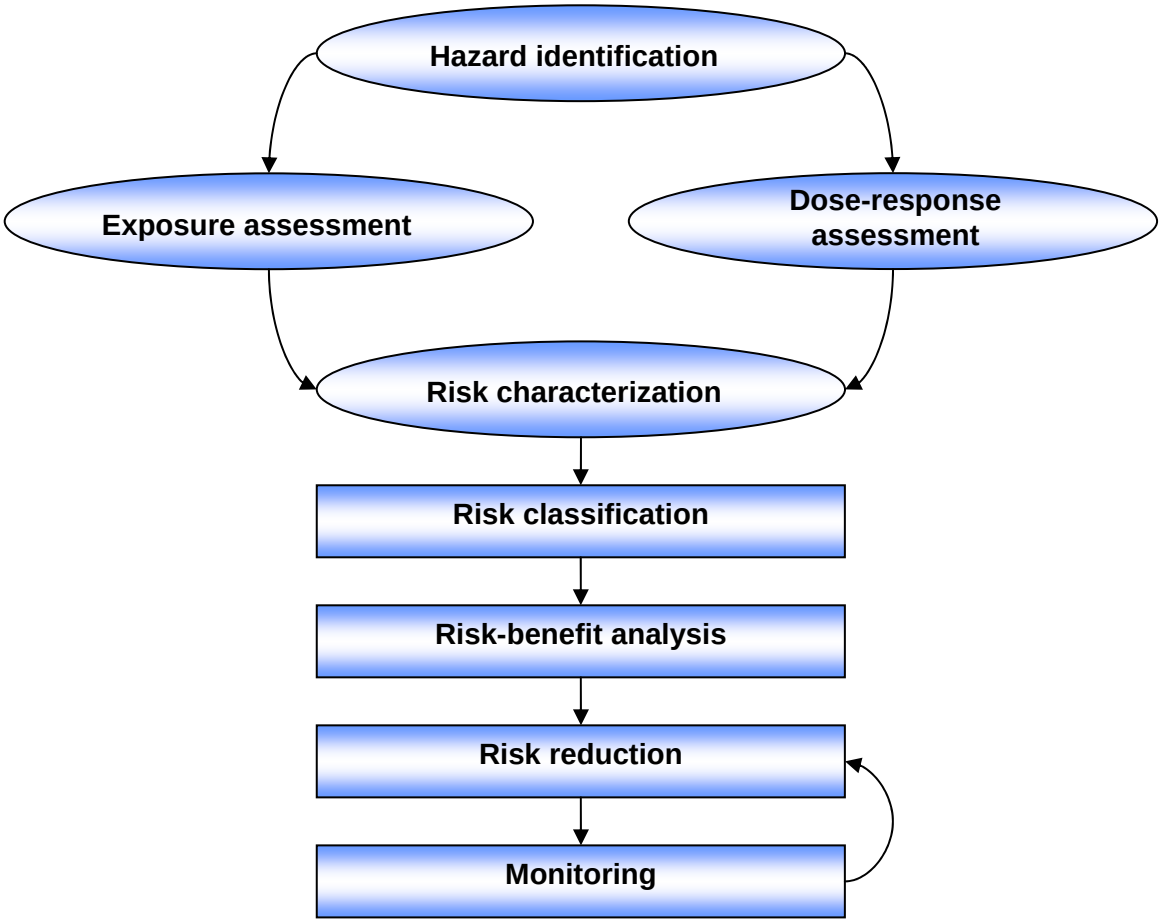

Figure 1.2 Steps in the risk assessment and risk management process

i) The hazard identification process is a systematic planning stage that identifies the important points that need to be covered by all subsequent steps in the HHRA. It identifies the adverse effects, inherently associated with environmental contaminants, it gathers and evaluates the data that describe whether the possibility of direct or indirect human exposure exists, and it points towards the possible health effects following exposure.

ii) Exposure assessment estimates the magnitude of actual or potential human exposure to a contaminant of concern, the frequency of this exposure and its duration. It is probably the most uncertain part of risk assessment because of the lack of information on emission factors during the production of chemicals (point-source emission) and about the use of chemicals in various products and their emissions (diffuse sources of pollution). The number of exposure pathways from environmental contaminants to the human body is numerous and variable. The routes of exposure are more easily distinguished: dermal contact, inhalation and ingestion. An important output of the exposure assessment step is the determination of the PEC, or predicted environmental concentration, which is used to estimate the total daily intake.

iii) Dose-response assessment characterizes the relation between the dose of an agent administered or received and the incidence of an adverse health effect. This step estimates the probability that an individual will experience negative health effects upon exposure to a chemical or a contaminated matrix. Data are usually obtained from animal laboratory studies, experimental field studies or less frequently from epidemiological studies of human populations. For most chemicals, laboratory derived no effect levels (NEL) are converted into predicted NELs or PNELs. Often, predicted no effect concentrations (PNEC) are derived. Another frequently used parameter in dose-response assessment is the No Observable (adverse) Effect Level (NOEL).

iv) Risk characterization integrates the exposure assessment and dose-response assessment into a quantitative and qualitative expression of risk. Basically, the PEC and the PNEC from the exposure and dose-response assessment respectively are integrated to give a risk quotient. This risk quotient will eventually serve as a basic parameter for the risk characterization.

These four corner stones form the basis for the risk management step. The subsequent step is the risk classification which is used to validate whether risk reduction is required. Decisions about risk classification are always related to decision about what risk level is acceptable. In order to obtain some kind of risk acceptability classification, two levels are distinguished: the maximum permissible level (MPL) and the negligible level (NL). An important differentiation should be made for compounds which do not have a treshold levels, such as carcinogens and mutagens. For these

compounds, a linear relationship is assumed between exposure (dose) and effect (incidence of cancer). Statistically, effects can therefore always occur. In such cases, the risk number is expressed as “maximal tolerable risk” or “negligible risk” and it represents the probability of additional cancer cases occurring. Once the risk classification process is completed, the risk-benefit analysis is carried out, which is basically a cost-benefit analysis. This step is multifactorial, since it needs to consider the technical feasibility of future risk reduction steps, the financial costs, social factors, legislative factors and multidisciplinary research. The outcome of the risk-benefit analysis leads to decisions about what risk reduction measures are taken to protect humans from the identified risks. With respect to chemicals that pose a threat to the environment or human health in particular, classification and labeling chemicals is required on the basis of their intrinsic properties. If it concerns contaminated sites or specific pathways of exposure, the operator, manufacturer or user is responsible for lowering the risk level to as low as reasonably achievable (ALARA). Safety and quality standards comprise guidelines, objectives and standards which assist in the risk reduction process. Well-known examples of these standards are Threshold Limit Values (TLV) and Acceptable Daily Intake (ADI).

1.3 Exposure routes for soil contaminants

Chemicals may reach the human body through soil, water, and air. These media serve as vehicles that carry chemicals to potential receptors. For soil contaminants specifically, the most important exposure pathways are direct soil contact, groundwater exposure and occupational exposure (e.g. construction workers). Besides these exposure pathways three direct exposure routes can be distinguished which refer to the ways that chemicals are transferred from environmental media into the body: inhalation, dermal contact and ingestion. For soil contamination in particular, all three routes are relevant but dermal contact and ingestion are the most likely exposure routes.

Inhalation

Human exposure to soil-borne contaminants by inhalation is less frequent than dermal contact or ingestion, but may occur when the chemical volatilizes from the soil (for example organic compounds) and is inhaled in the vapor phase, or when soil particles as such, containing the chemical, are respired (NRC, 2003). When contaminants as such or their particulate carriers are inhaled and pass the nose/mouth, they reach the pulmonary tract which consists out of an upper part, the tracheobronchial region, and a lower part, the alveolar region. The tracheobronchial

region is composed of a layer of cilia and mucus and an epithelium of 50 µm thick, followed by the blood capillaries. The rate of contaminant absorption in this part of the pulmonary tract is low since it takes more time for the contaminants to cross the thick epithelium. This longer residence time however also allows for the metabolism of inhaled chemicals and is responsible for the fact that 95% of all lung cancers are located in this region of the lungs. The alveolar region with the alveoli contains an epithelium which is only 5 µm thick and which is followed by the blood capillaries. This thin epithelium causes contaminants to be rapidly absorbed across the epithelium upon which they reach the blood stream through the capillaries.

Soil-bound contaminants which have a low vapor pressure may volatilize from the soil or groundwater by desorption of the chemical from the soil matrix, movement from the soil pores to the soil surface and mixing with the atmosphere. This process is largely dependent on the contaminant vapor pressure, the concentration of the contaminant in the soil with depth and the interaction of the compound with the soil matrix by sorption (Cockerham and Shane, 1994). Best known examples are the BTEX compounds (benzene, toluene, ethylbenzene, xylene) and other volatile organic compounds. If contaminants are inhaled as vapors, they are assumed to be 100% bioavailable.

Respirable particles are considered to be smaller than 10 µm and have been estimated to make up 30 to 50% of the total suspended air particles (Paustenbach, 1988). About 50% of these particulates may be deposited in the tracheobronchial region. Subsequently, they move upwards by mucociliary activity and are eventually swallowed and taken up in the gastrointestinal tract. Exposure of these initially inhaled particles is therefore occurring through the gastrointestinal tract. The remaining particles may reach the alveoli. Particulate material can be divided in to PM-2.5 particles, fine particles smaller than 2.5 µm in diameter, and PM-10 particles, coarse particles smaller than 10 µm in diameter. Combustion products from motor vehicles, the industry and residential fireplaces usually give rise to fine particles, whereas coarse particles arise from traffic, construction sites, wind erosion and material handling. In general, the inhalation of particles is fairly relevant for organic compounds with low vapor pressures such as polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAH) (NEPI, 2000).

Dermal contact

The skin protects the human body from external influences by an outer epidermis which consists of a non-viable cell layer, the *stratum corneum* and an inner layer consisting of viable cells which have a metabolic capability. The latter is particularly relevant for certain PAHs, such as benzo(a)pyrene, which can be metabolized by

these cells (NEPI, 2000). Uptake of contaminants by the skin is merely a diffusion process with the thickness of the skin, the chemical properties of the contaminant and the concentration on top of the skin being the most important variables determining the bioavailability of contaminants following dermal exposure. The human health risks associated with dermal exposure to soil-bound contaminants are the result from the direct toxic effects of the contaminant on the skin or by absorption of the compound through the skin and subsequent systemic toxicity. The dermal bioavailability of soil contaminants can be compared to oral bioavailability (see part 2.5) since it also consists of two parts: chemical availability (bioaccessibility) and absorption. Bioaccessibility describes the fraction of the chemical that desorbs from the soil matrix and is available for absorption by the skin, whereas absorption describes the transport of the chemicals across the skin biological membranes to the blood stream (NEPI, 2000).

Ingestion

Because the gastrointestinal (GI) tract is the principal site of nutrient uptake, it is a prime location for uptake of contaminants as well, not only soil-bound contaminants but also hazardous compounds in nutrition, drinking water or ingestion of contaminants from children's toys. Although the transport of contaminants across biological membranes is fairly similar for the different exposure pathways, the digestive processes inside the GI tract result in more complicated transport phenomena than with inhalation or dermal contact. For example, a colloid- or particle-bound contaminant can reside in the GI tract for hours to days which will affect the particle-contaminant associations in the intestinal lumen to a large extent. Additionally, the absorption processes are more complicated: besides diffusion processes of simple organic compounds, specific ions can also be transported as well as non-charged elements that are associated to for instance amino acids (NEPI, 2000). This complexity of oral exposure to environmental contaminants will be discussed below.

2. Oral exposure to contaminated soil

2.1 Ingestion

Oral exposure to soil-bound contaminants mainly occurs by ingestion of soil and dust particulates and consumption of badly cleaned vegetables. In HHRA, typical human soil ingestion rates are estimated between 1 and 500 mg d^{-1} (Brunekreef et al., 1987; Vanwijnen et al., 1990; Calabrese et al., 1997a, 1997b; Stanek et al., 1997). Site-specific risk assessment studies incorporate soil ingestion with a maximal daily intake of 50 and 150 mg soil d^{-1} for adults and children, respectively (EPA, 1997). Soil

ingestion may seem rather unlikely for adults, yet the pathway is more indirect. Depending on the profession or the weather situation, soil particles may end up in the gastrointestinal tract. For example, construction workers may get exposed to soil and dust particulates via the inhalatory route, especially under windy and dry weather conditions. As discussed earlier, particulates that are too large to reach the alveolar region of the inhalatory tract, may migrate up to the pharynx again through mucociliary movements. These soil particles may then be ingested and eventually reach the intestinal lumen. It is estimated that construction workers are exposed to 300 mg soil d^{-1} on average (EPA, 2001). Likewise, the soil surface from urban areas is more prone to atmospheric depositions from diesel exhaust particles, PAHs from car tires and several combustion processes. Especially in densely populated or industrial areas, this indirect ingestion route of soil particulates may reach significant values. In addition, ingestion of soil particles also occurs by vegetables or fruits that are grown in the garden. It is estimated that the soil loads for vegetables with leafy tissues is 20 mg soil kg^{-1} DW (dry weight) after normal washing and for fruits 2 mg soil kg^{-1} DW. However, higher soil ingestion rates are also encountered, primarily then by small children that ingest larger amounts of soil due to their hand-to-mouth behavior. Estimated soil intake rates by small children are between 100 and 500 mg d^{-1}. Direct soil ingestion may even impose a larger health risk to *pica*-afflicted children, who, due to their unusual hand-to-mouth behavior, occasionally ingest several grams of soil with reported intakes between 5 and 60 g d^{-1} (Calabrese et al., 1999).

These examples of incidental soil ingestion are important pathways of contaminant exposure in HHRA. In its basic form, the intake equation for incidental ingestion of soils is (NRC, 2003):

$$Intake = \left(\frac{C_s \times IR \times RAF}{BW} \right) \left(\frac{EF \times ED}{AT} \right)$$

where:

C_s = chemical concentration in the soil at the point of contact

IR = incidental ingestion rate of soil

RAF = relative absorption factor

BW = Body Weight

EF = Exposure frequency

ED = Exposure duration

AT = attenuation time (period over which exposure will be averaged)

To exemplify, the intake of lead from a contaminated soil (500 mg lead kg^{-1} soil) by children can be calculated using scenario-specific values and default values as

proposed by the EPA (EPA, 2001). Default values for soil ingestion by children are a soil ingestion of 200 mg d^{-1}, body weight 15 kg, 350 d yr^{-1} exposure frequency, 3 yr exposure duration and an attenuation time of 6 yr. The relative absorption factor considers the fact that not 100% of the lead will be intestinally absorbed because a fraction is still bound to the soil matrix. If an RAF of 60% is chosen, then the incidental lead ingestion by children is calculated as:

$$Intake = \left(\frac{500 \times 0.2 \times 0.6}{15}\right)\left(\frac{350 \times 3}{6}\right) = 1.9 \frac{\mu gPb}{kgBW * d}$$

In general, human health risk assessment models assume that 100% of an ingested contaminant is absorbed and taken up in the systemic circulation. However, the availability of contaminants ingested with soil is an important variable significantly affecting soil ingestion exposure factors (Palumbo and Klinck, 2002). Researchers have indicated that current risk assessment models are not reliable by demonstrating that the actual absorption of soil-bound contaminants is substantially lower than 100% (Williams et al., 1998; Pu et al., 2003). The oral bioavailability process with regards to soil ingestion will be discussed below (part 2.5).

2.2 Human gastrointestinal tract

The primary function of the GI tract is to degrade ingested food particles to this extent that smaller, individual molecules can be more easily absorbed and taken up in the blood circulation. In this way, complex sugars of different polymerization and substitution degree can be broken up in single sugar molecules, proteins are split into amino acids and fats are converted to triglycerides and fatty acids. Besides that, vitamines and trace elements make up a smaller, but crucial part of the nutritional composition. The GI tract roughly measures 8 meters from the mouth to the rectum and is basically the ‘inner outside’ of the human body (Figure 1.3). Ingested components are only taken up by the body if they are digested into molecules small enough to be intestinally absorbed. Food compounds are first mechanically broken up by chewing from the mouth. This entails that the cell walls from ingested plant tissues are destroyed, exposing the cell content more easily to the digestive juices such as saliva, gastric juice, intestinal juice, bile and pancreatic juice. Absorption of available nutritional compounds primarily takes place in the small intestine, whereas the residual, non absorbed compounds are transferred to the large intestine, further digested by the present microorganisms and eventually excreted with the feces.

Mouth. The mouth is the first place of enzymatic digestion of food compounds. The secreted saliva contains enzymes that convert polysaccharides to maltose and the latter to glucose molecules. Good chewing is therefore very important in the digestion of starch which makes up an important amount of our daily food intake. A good mixture of the food with saliva also ensures an easier swallowing and transport to the esophagus, which is the least important part of the GIT, connecting the mouth with the stomach.

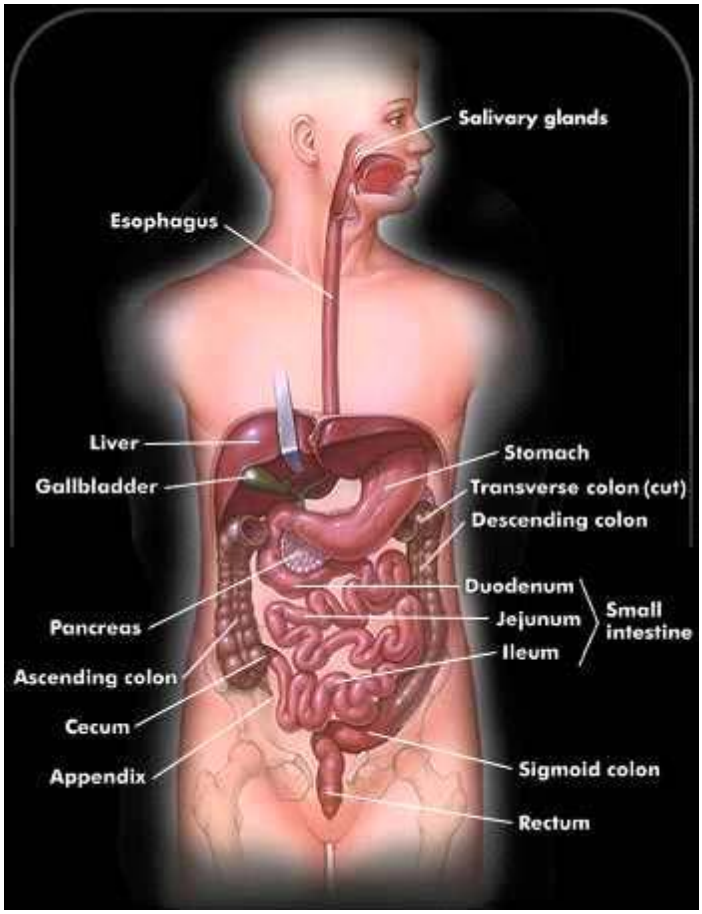

Figure 1.3 Overview of the gastrointestinal tract and relevant organs

Stomach. The stomach is a widened part of the GIT, consisting of 4 parts: the cardia, the corpus, the fundus, and the pyloric antrum, the latter giving access by the pyloric sphincter to the duodenum. An empty stomach is characterized by an open pyloric sphincter and relatively little gastric juice, mainly consisting out of water and mucin which is secreted by glands in the stomach wall. The ingested food that enters the stomach via the cardia, is grinded in the corpus, while the fundus serves more as a storage. The ingestion of food also results in a higher secretion of gastric juice which changes in composition by the higher concentration of hydrochloric acid and digestive enzymes. The hydrochloric acid solubilizes food components and has an important bactericidal effect towards microorganisms that are taken up with the nutrition. A

mucus layer covers the stomach wall to protect the epithelium from the acidic environment inside (pH 1 to 2). The acidic environment is also crucial in the activation of the digestive enzyme pepsin, which has proteolytic activity in an optimum pH range of 1 to 3 (Ganong, 1997). Hormones are also involved in the stomach digestive processes. Gastrin stimulates the hydrochloric acid production in the stomach, whereas secretin also promotes the pancreas to secrete digestive enzymes. After a certain residence time, the chyme from the stomach is gradually released to the duodenum by intermittent opening of the pyloric sphincter and the peristaltic movement from the small intestine.

Small intestine. The small intestine is the most important part of the GI tract. The human food digestion which is initiated for starch in the mouth and for proteins in the stomach, comes to completion in the small intestine, which is about 5 meters long and can be separated in the duodenum, the jejunum and ileum (Charman et al., 1997; Hidalgo, 2001). The proximal part, the duodenum, starts from the pyloric sphincter and is about 30 cm long. It receives the secretions of the pancreas and the liver, i.e. the bile via the gal bladder. The pancreatic juice and bile play a crucial role in the further digestion of the semi-digested food from the stomach. The strong acidic chyme from the stomach contains mainly maltose and glucose from starch, other more complex sugars, polypeptides and smaller protein fragments and lipids, which are not yet digested in the stomach. The alkalic pancreatic juice neutralizes the acidic stomach content to a pH of 7.5 and several pancreatic enzymes such as amylases, proteases and lipases are responsible for the further food digestion process. The enzymatic conversions in the small intestine entail the conversion of sugars to single sugar molecules, the breakdown of peptides to their individual amino acids and the conversion of lipids to fatty acids. The latter process, which is carried out by pancreatic lipases, is stimulated by the secretion of bile, produced in the liver and stored in the gal bladder.

The secreted bile does not contain enzymes but consists out of bile salts which act as an emulgator to distribute large fat globules to small fat droplets. This process of emulsification makes the lipids more accessible to the pancreatic lipases so that the digestion efficiency of lipids is increased. The chyme is transported from the duodenum to the jejunum and ileum through peristaltic movements. Although the jejunum and ileum are the main site of absorption of nutrients and electrolytes, they also have a digestive function as the epithelial wall secretes digestive juices, which contain enzymes that finish the digestion process: small peptides are converted to their amino acid building blocks and remaining disaccharides are converted to the monosaccharides glucose, galactose and fructose. These basic food components are

the compounds which are then available for intestinal absorption. Non-absorbed food compounds are further transported through the GI tract and reach the large intestine.

Large intestine*.* The large intestine receives the chyme that is poor in components with direct nutritional value, since the latter are absorbed in the small intestine. From the food digestion perspective, the primary function of the large intestine is to absorb most of the water from the chyme and the remaining electrolytes. The colon absorbs sodium by an active carrier and chloride in exchange for bicarbonate (Cummings et al., 2004). Potassium is the dominant cation excreted in fecal fluid. The absorption of electrolytes creates a difference in osmotic pressure over the colon epithelium resulting in the absorption of 90% of the water in the chyme (Sandle, 1998). In contrast to the small intestine, which contains limited amounts of bacteria, the large intestine is heavily colonized with microorganisms: more than 400 different microbial species are present in concentrations of 12 log CFU/g. This is roughly about ten times as much as the amount of human cells in the body. It is now recognized that the metabolic potential of the human colon microbiota is extremely large, rivaling that of the liver in the number of biochemical reactions and transformations in which it participates (Macfarlane and Macfarlane, 1997). Given the absence of oxygen in the large intestinal lumen, the microbial community typically performs fermentation processes. The main microbial transformation processes are carbohydrate and protein fermentation, transformation of bile salts and steroids and the metabolism of xenobiotic compounds (Gibson and Williams, 1999). Besides that, the microbial community also plays a critical role in the immune system and may (in)activate potential mutagenic metabolites. The average residence time in the large intestine is much longer (36 to 48 hours) than in the small intestine (6 to 8 hours). Besides the normal absorption of water and elektrolytes, the colon epithelial wall may also absorb microbial fermentation products and other metabolites.

2.3 Intestinal transport

2.3.1 Morphology

The digestion process in the GI tract would be useless if the basic nutritional compounds could not be absorbed and transported across the intestinal wall. The absorption of compounds is however controlled by the mucosa which lines the luminal wall of the GI tract, hence functioning as a selective permeability barrier (Hidalgo, 2001) permitting the absorption of nutrients such as sugars, amino acids, peptides,

lipids and vitamins (Strum, 1981; Said et al., 1987) and limiting the absorption of xenobiotics, digestive enzymes, and bacteria. The predominant site of absorption is the jejunum and ileum where the mucosa can be divided into three distinct layers: the muscularis mucosa (the deepest layer), the lamina propria (bounded by the epithelium above and the muscularis mucosa below) and the uppermost single-layered epithelium (Doherty and Charman, 2002). The lamina propria is a structural support for the epithelium, containing lymph vessels, nerves, lymphocytes, macrophages that attack foreign compounds that enter this layer from the GIT and blood vessels for nourishment of the epithelium.

The epithelium is in direct contact with the chyme (intestinal suspension). Intestinal absorption is facilitated by several modifications that increase the absorptive surface area of the small intestine to 150-200 m^2 (De Sesso and Jacobson, 2001). The large surface of the intestinal wall is established by the formation of circular folds (Kerckring folds or *plicae circulares*) and by folding of the intestinal epithelium itself, called villi, fingerlike formations of 1 mm long in the intestinal lumen (Figure 1.4A). Each villus contains blood and lymphatic capillaries by which absorbed compounds are transported to the liver (portal vein) or the rest of the body. The surface of each villus consists of different cell types such as goblet cells, which secrete mucin, endocrine cells and enterocytes, which are absorptive cells. The enterocyte is the most predominant cell type in the villi and is characterized by the presence of small microstructures, microvilli that are 1 µm long and 0.1 µm wide (Figure 1.4B). The presence of these microvilli significantly contributes to the large extension of the available surface area for intestinal absorption.

2.3.2 Types of transport

Ingested and digested food components, drugs or environmental contaminants can become available for transport across the intestinal epithelium. Depending on the type of compound, different types of absorption can be distinguished (Figure 1.5).

Firstly, paracellular transport is the aqueous extracellular absorption of compounds through the tight junctions that separate the enterocytes. This type of transport is reserved for small, hydrophilic molecules that can easily migrate between two enterocytes by the process of diffusion (Hidalgo, 2001). The other types of intestinal transport all occur through the enterocyte and can be grouped as transcellular transport, which entails transport across the cell membrane itself. The surface area of the transcellular route is much larger than that of the paracellular route (99.9% versus 0.01%). Hence, compounds that are only transported through the paracellular pathway

have a low absorption, whereas compounds that are readily transported across the cell membrane have a high absorption (Pade et al., 1997).

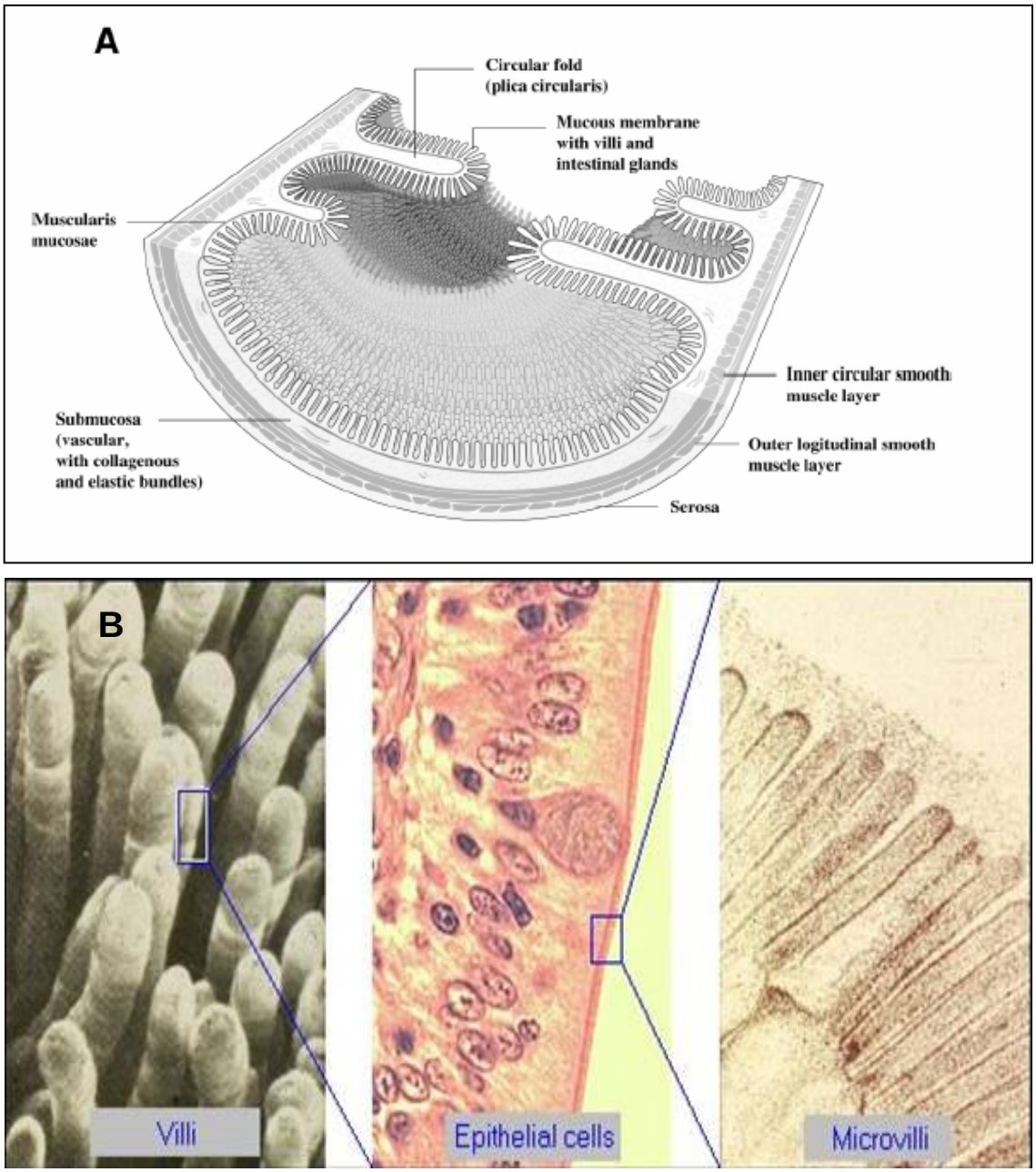

Figure 1.4 Scheme of the ileum luminal structure with the circular folds and villi (A) and magnification of the villi to show the enterocytes and microvolli structures (B)

The simplest mechanism of transcellular transport is passive diffusion by which molecules migrate through the enterocyte down a concentration gradient. Products of fat digestion, hydrophobic vitamins and other hydrophobic compounds such as organic contaminants or certain drugs may be incorporated in mixed micelles with bile salts,

which diffuse to the brush border where they are absorbed by the lipid membrane of the enterocytes (Koldovsky et al., 1995; Hack and Selenka, 1996; Oomen et al., 2000).

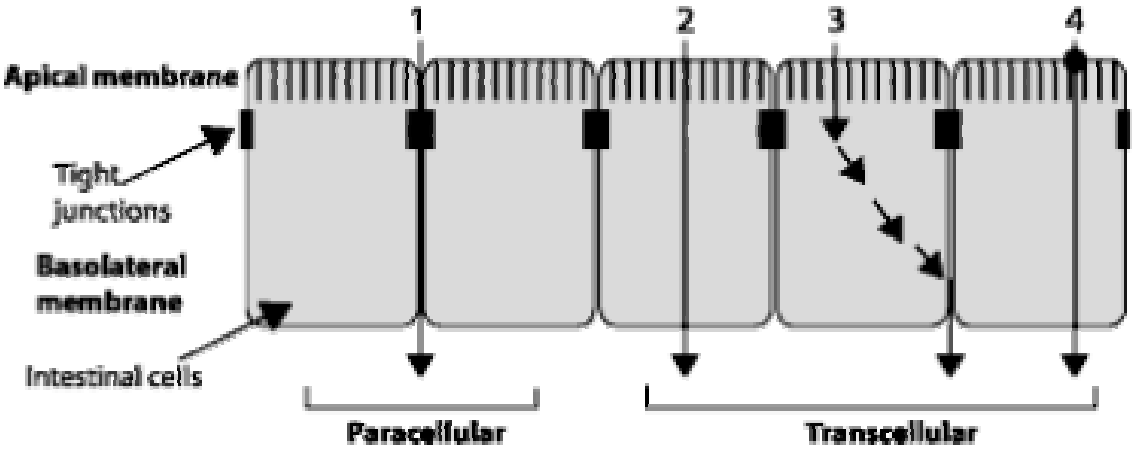

Figure 1.5 Schematic representation of the different intestinal transport routes across the epithelium. 1: paracellular transport, 2: passive diffusion, 3: transcytosis, 4: carrier mediated transport

However, specialised transport systems that make use of carrier proteins, may also affect the absorption rate of specific compounds by either facilitated diffusion (no energy required), active transport (energy-consuming) or even specific efflux transport (Tsuji and Tamai, 1996). In this way, certain compounds may exhibit absorption values that are different from the expected values that are based on their intrinsic membrane permeability properties. The small intestine expresses several types of carrier proteins which are responsible for the transport of nutrients such as hydrophilic dipeptides or vitamins. Additionally, some of these carrier proteins are also believed to be involved in the transport of drugs or specific xenobiotic compounds.

In contrast to this carrier-mediated transport, there is also carrier-limited transport which mediate the transport of compounds from the enterocyte cytoplasm to the intestinal lumen. This process is known as apical efflux. The best-studied efflux carrier protein is the P-glycoprotein which is known to limit the intestinal absorption of a large number of drugs (Sparreboom et al., 1997; Dautrey et al., 1999; Stephens et al., 2001). Currently, two genes have been described that code for this apical efflux activity: *mdr*1 and *mdr*2 (MDR: multiple drug resistance) (Chin et al., 1993). MDR1 gene products would be responsible for the drug resistance that have been described in several cancer cells (Juliano and Ling, 1976) and other tissue including the liver, kidney, pancreas and intestinal epithelium (Thiebaut et al., 1987), whereas MDR2 gene products would primarily occur in the liver.

The final transport mechanism by the enterocytes is the vesicular transport which may enhance the absorption of xenobiotic compounds. This type of transport basically consists of the formation of invaginations of the enterocyte plasmamembrane which pinch off and form intracellular vesicles that surround the absorbed compound.

Absorbed compounds from the small intestine that escape biotransformation by the enterocyte, are usually transported to the liver where they can be subjected to liver biotransformation processes, after which they may reach the target organs or be excreted from the body by the urinary and biliary tract.

2.3.3 Permeability of colonocytes versus enterocytes.

Although the morphology of the large intestine, or colon, is substantially different from that of the small intestine, the colon is lined with epithelial cells, colonocyctes, which allow the absorption of components across the intestinal wall too. Non-digested food components, digestion products, secreted human metabolites and microbial fermentation products may (partly) become available for absorption. Hence, the small intestine is not the only site of absorption. This is of importance for colon specific drug release. The colon microorganisms produce a large number of degrading enzymes that enable to degrade polymers that are used to encapsulate drugs. This microbially controlled delivery of drugs to the colon is independent of pH variations along the GI tract or enzymatic processes, since the drug, peptide, or protein is only released upon arrival of the dosage form at the colon (Zhang et al., 2002).

The colon also harbours a vast microbial community with an enormous metabolic potency. Ingested contaminants or xenobiotic compounds in general may thus be biotransformed by the indigenous microbial population, leading to metabolites that may become available for absorption. Hence, risk assessment studies that monitor the bioavailability of ingested contaminants, also need to consider the fraction absorbed from the colon and their metabolites. Although the available surface area for colonic absorption is much smaller than that of the small intestine, it may be of significant importance given the possibility that hazardous microbial metabolites may be produced that pose a risk to the colon epithelium.

In general, the permeability to hydrophylic compounds decreases once the small intestine is passed. This can be attributed to the smaller surface area of the colon membrane and the increased tightness of the epithelium (Ungell et al., 1998). However, this decreased permeability is not always observed with hydrophobic compounds. Depending on the type of compound and the species of origin, membrane transport studies show higher permeability to hydrophobic compounds in the colon

than in the small intestine, and *vice versa*. A sound explanation for these differences is not yet available but one of the reasons may be that epithelial enzymes that are involved in metabolic conversion processes, are responsible. A second reason may be the presence of efflux carrier proteins (p-glycoprotein) in the colon epithelium, thus interfering with the absorption process. It may be interesting to report that comparative *in vitro* permeability studies with small intestine and colon epithelia revealed that the colon epithelium is more permeable to the natural estrogen 17β-estradiol than the small intestine epithelium (van der Bijl and van Eyck, 2003). In conclusion, identifying the different transport mechanisms for specific compounds of interest, such as ingested contaminants, is extremely important for assessing the internal dose of these compounds in risk assessment studies.

2.4 Biotransformation of xenobiotics

Many organic contaminants in the environment are xenobiotic or in other words, foreign to the human body or the natural environment in general: drugs, carcinogens, pesticides, food contaminants, toxins… Many xenobiotic compounds are highly hydrophobic and tend to accumulate in the body where they may potentially exert toxic effects. The human body has therefore developed a defense mechanism, called biotransformation, which has as main purpose to convert these xenobiotic compounds to more hydrophilic compounds so that they can be more easily removed from the body. Biotransformation of ingested components in the gut wall and gut lumen is of significant importance to the biological availability of those xenobiotics and hence to their potency to become biologically active in the body. Metabolism in the gut wall during intestinal absorption or in the liver may lead to the elimination of the xenobiotic compound before it reaches the systemic circulation. Metabolism in the gut lumen is also possible and is carried out by intestinal microorganisms. Here, the amount of the ingested compound which is available for intestinal absorption may be modulated by detoxification processes, but also toxification processes. In summary, the biotransformation of ingested xenobiotics can thus be divided into human and microbial metabolism.

2.4.1 Human biotransformation activity

Most ingested xenobiotic compounds that undergo intestinal absorption are subjected to enzymatic activity from the human body. Although the liver is the prime site of biotransformation activity, every organ or tissue in the body is capable of performing biotransformation reactions: skin, lungs, heart, kidneys, bone marrow, brains… (Connely and Bridges, 1988). The process of inactivating ingested contaminants after oral absorption and before entering the systemic circulation is referred to as first-pass effect (Klaassen, 1986). Especially the enterocytes, the cells from the intestinal epithelium, play a significant role in the biotransformation of ingested and subsequently absorbed components, provided that suitable enzymes are present in the enterocyte. This entails that biotransformation in the enterocytes can only occur during transcellular transport (Doherty and Charman, 2002). The extent of such first-pass metabolism depends partly on enzyme concentration (Klippert et al., 1982) and also on the rate of drug transfer through the mucosal cells (Borm et al., 1985). The highest concentration of biotransformation enzymes in the intestinal epithelium can be found in the tips of the villi whereas their content decreases towards the crypts (Guengerich, 1997). The metabolic activity is usually higher in the duodenum and jejunum compared to the ileum and colon. Yet, this can not be generalized, as evidenced by data that showed that 2-aminofluorene acetylase activity in the colon and duodenum epithelial wall are not different (Ilett et al., 1990). Research on the gene expression of cytochromes P450 in different parts of the human gastrointestinal tract revealed that specific cytochromes P450 are indeed more present in the small intestinal enterocytes, but that other cytochromes P450, such as CYP 1B1, is better represented in the colonocytes, particularly from the ascending colon (Table 1.1) (Finnström, 1998).

Table 1.1 Occurence of Cytochrome P450 (CYP) biotransformation complexes as a function of their human intestinal localisation

CYP	Duodenum / jejunum	Ascending colon	Transverse colon	Descending colon
1B1	+	++++	+	+
2E1	++	++	+	++
3A4	++++	++	+	++
3A5	+++++	++++	+++	++

Non-biotransformed xenobiotic compounds that have been absorbed from the intestinal lumen, may then be transported via the portal vein to the hepatocytes of the liver. Here, they may be biotransformed which may result in the excretion of the compound via the bile in the duodenum. Biotransformation reactions were previously thought to induce detoxification of the ingested contaminants, yet it has become clear that biotransformation enzymes may also 'bioactivate' compounds, resulting in stable or unstable metabolites that are more toxic than the parent compound. Unstable intermediates generally exert their toxicity in the tissue(s) where they are produced, whereas stable ones may be formed in one tissue only (usually the liver), released into the bloodstream and then affect other tissues (Connelly and Bridges, 1988). In several instances there are competing metabolic pathways between activation and detoxification of a foreign chemical. Whether a toxic reaction will be initiated in such situations will depend on the relative balance between these pathways (Dybing et al., 2002).

Basically, the human biotransformation process can be split up in two major steps: phase I, the functionalization reactions including oxidation, reduction and hydrolysis) and phase II, the conjugative reactions (Nebbia, 2001).

Phase I reactions usually result in the introduction of functional groups (-OH, -NH_2, -SH, -COOH) on the xenobiotic molecule, which makes it more easy to conjugate the compounds with endogenous substrates (Gibson and Skett, 1994). Several enzymes are involved in phase I reactions: alcohol and aldehyde dehydrogenases, xanthine and amine oxidases, aromatases, and hydrolysis initiating enzymes. Enzymes from the cytochrome P450 (CYP) superfamily are represented the most. CYP enzymes that are involved in the metabolism of organic chemicals are predominantly from the CYP1, CYP2 and CYP3 subfamilies, which can be further divided in isoenzymes with affinity for specific compounds. Isoenzymes that have been detected in the enterocytes from the small intestine are CYP1A1, CYP2C, CYP2D6 and CYP3A4, the latter accounting for 60% of the phase I enzymes in the enterocytes and responsible for metabolization and inactivation of a large number of compounds (Hebert et al., 1992; Doherty and Charman, 2002). Additionally, the apical efflux activity from P-glycoproteins seems to be coregulated with the CYP3A4 enzyme (Wacher et al., 1995). This way, the P-glycoprotein efflux pump contributes more efficiently to the inactivation of xenobiotics. In general, CYP mediated oxidation reactions can be regarded as the most important pathway by which bioactivation of compounds occurs. Benzo(a)pyrene, one of the most dangerous PAH species, is for example biotransformed by CYP1A enzymes to 7,8 epoxide derivatives which are potent pro-carcinogens (Stroomberg, 2002).

Although phase I biotransformation reactions may sometimes lead to the bioactivation of xenobiotic compounds, they are an important prerequisite to phase II reactions, since the phase I metabolites are good substrates for conjugation enzymes, which effectively detoxify the initial metabolite. Yet, phase II biotransformation reactions may also occur with xenobiotics that have not gone through a phase I step. The resulting conjugates are much more hydrophilic than their precursors and may hence be easily removed from the body via biliary or urinary excretion. Chemicals which are less hydrophobic and show intermediate polarity may only be subjected to phase II enzymes and not phase I.

The human intestinal epithelium has been described to contain relatively high levels of phase II enzymes, mostly glucuronyltransferase, N-acetyltransferase, sulfotransferase and glutathion-S-transferase (Table 1.2). Xenobiotics excreted into the bile as glucuronide conjugates, may re-enter the circulation after being hydrolysed by glucuronidase activity from gut microbiota. This process is called enterohepatic circulation (Dybing et al., 2002).

Table 1.2 Typical phase I and phase II biotransformation enzymatic families or enzymes (Source: WHO, 2002 IPCS training)

Enzyme	**substrate**
Cytochrome P450 1A1	Polycyclic aromatic hydrocarbons
Cytochrome P450 1A2	Aryl and heterocyclic amines
Cytochrome P450 2A6	Coumarin
Cytochrome P450 2C19	Drugs (diazepam, omeprazol…)
Cytochrome P450 2D6	Drugs
Cytochrome P450 2E1	Ethanol, organic solvents, nitrosamines, paracetamol
Cytochrome P450 3A5	Chlorinated compounds
N-acetyltransferase	Aryl amines
Sulfotransferase	Aryl and heterocyclic amines
Glutathion S-transferase	Polycyclic aromatic hydrcarbons, oxides and diol-epoxides

2.4.2 PAH: example compound for human biotransformation

In view of the research that will be described in this dissertation, it is recommended to give a short description of the human biotransformation processes for polycyclic aromatic hydrocarbons (PAH). PAHs form a group of chemicals of concern that are generated during the combustion of fossil fuels and other organic fuels. It is assumed that anthropogenic combustion activities are responsible for more than 90% of the polycyclic aromatic hydrocarbons (PAH) that are released in the environment. Together with the persistent organic pollutants (POP) such as heterocyclic amines, polychlorinated biphenyls, dioxine-like chemicals and certain pesticides, PAHs are considered to be one of the most hazardous xenobiotic compounds in the environment to which the human can be exposed. The toxic effects include cancer, immunologic compromise, birth defects, endocrine disruption, genotoxicity… The fundamental problem with these compounds is their persistence in the environment and their resistance to photocatalytic breakdown, chemical and biological degradation in the atmosphere, soil and water phase (Schafer and Kegley, 2002). These recalcitrant properties explain their long residence time and stability in the environment and also their bioaccumulation in biological tissues and biomagnification throughout the entire foodchain.

A common characteristic of PAHs, similar to polychlorinated biphenyls (PCB), polychlorinated dibenzo-furanons (PCDF) or -dioxins (TCDD), is their binding affinity for the human aryl hydrocarbon receptor (AhR), once they are taken up in the human body. This triggers several biotransformation processes which result in the detoxification and excretion of the compounds from the body, or their possible bioactivation and bioaccumulation which give rise to several toxic effects.

2.4.2.1 Role of the aryl hydrocarbon receptor

The intracellular mechanism by which PAHs are biotransformed in the body and subsequently exert their toxic effects, has been thorougly investigated (Birnbaum, 1995; Whitlock et al., 1996; Seidel et al., 2000). When reaching the enterocytes or hepatocytes or other cells with biotransformation capacity, these compounds may act as ligands to the aryl hydrocarbon (or dioxin) receptor (AhR) protein in the cytoplasm (Figure 1.6), which has been described to play a central role in the toxic response of specific aromatic hydrocarbons by the regulation of typical human biotransformation enzymes.

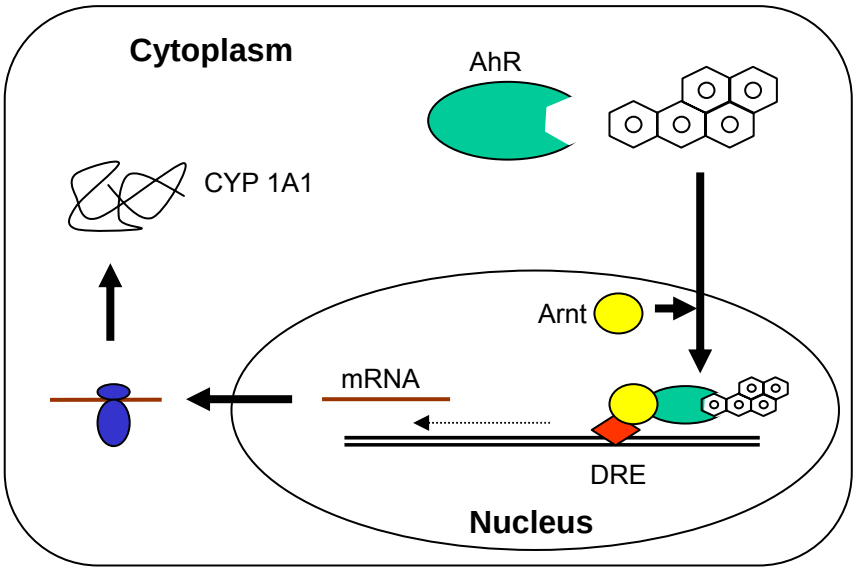

Figure 1.6 Binding of benzo(a)pyrene as model PAH compound to the AhR, migration to the cell nucleus, binding to the DNA responsive element (DRE) in the promotor region, transcription and CYP 1A1 protein synthesis.

Together with the aryl hydrocarbon receptor nuclear translocator (Arnt), the AhR is part of a heterodimeric transcription factor called the aryl hydrocarbon receptor complex (Hankinson, 1995; Schmidt and Bradfield, 1996). When activated by a ligand – the PAH molecule – the AhR is subjected to a conformational change which exposes a nuclar localization sequence. The AhR-ligand complex is then transferred to the cell nucleus (Denison et al., 2002) where it dissociates from the protein complex and binds to the Arnt (Figure 1.6). The Arnt-AhR heterodimer formation results in the transformation of the AhR-ligand complex to its DNA-binding form (Hankinson, 1995). This subsequently binds to DNA responsive elements (DRE) in the promotorregion of genes which are responsible for the production of cytochrome P450 1A1 enzymes (Poland and Knutson, 1982) in the case of environmental contaminants such as TCDDs and PAHs. It is generally assumed that AhR binding appears to be essential for toxicity, although non-AhR binding mechanisms have not been ruled out (Birnbaum, 1995). The regulation of other genes and geneproducts by ligand activation of the AhR is similar and has been reported for CYP 1A2, CYP 1B1, NADPH quinon reductase and glutathion-S-transferase.

2.4.2.2 Health effects from PAHs

Acute toxicity. There are only limited reports on the acute oral toxicity of PAHs. In general, PAHs are highly lipophilic and are therefore easily accumulated in the phospholipid bilayer of cell membranes. This harms the cell membrane integrity and may lead to nonpolar narcosis. However, such significant acute toxic effects from

PAHs are only caused at high, rather unrealistic concentrations. Naphthalene turns out to be the most acute toxic PAH compound with LD50 values ranging from 354 to 1200 body weight (BW) for mice and guinea-pigs respectively whereas the LD50 values for fluoranthene and anthracene were estimated around 2000 and 18000 mg kg^{-1} BW respectively in rat and mice experiments (Smyth et al., 1962; Shopp et al., 1984; Sax and Lewis, 1984; Montizaan et al., 1989).

Carcinogenesis. The real health hazard from PAHs comes from the potency of these compounds to bind the AhR and thus promote the production of biotransformation enzymes which may bioactivate the PAH compounds, leading to more toxic intermediates than the parent compounds. The activation of the AhR is considered the most important parameter for the carcinogenic potency of not only PAH, but also PCB, TCDD, PCDF… (Cheung et al., 1993). PAHs thus have a promotor function since they interfere with gene expression systems and are therefore epigenetic carcinogens. The purported effects from epigenetic carcinogens are usually resulting from long or very high exposures. However, PAHs may also react as genotoxic carcinogens. One single exposure to a genotoxic substance may already be sufficient to induce tumour formation.

Biotransformation of PAHs usually leads to the addition of hydroxyl groups on the PAH molecule. The phase II conjugation of the hydroxy-PAH with a glucuronide- or a sulphate-group facilitates the excretion with bile or urine (Figure 1.7). In contrast to this, the hydroxylation of the PAH molecule may also lead to the formation of epoxides (James and Boyle, 1998; Penning, 1993). Epoxides are highly reactive because the internuclear bond angles are smaller compared to normal sp^3 bond angles. The epoxides may be hydrolysed by the enzyme epoxide hydrolase to form dihydrodiols. Reaction with dihydrodiol dehydrogenase then leads to a stable diol that can be conjugated and excreted (Penning, 1993). However, some dihydrodiols go through a second epoxidation to yield a diol-epoxide. This diol-epoxide can react with macromolecules, such as proteins or DNA (Figure 1.7), to covalently bind to cellular DNA and form adducts with guanin or adenin (Jeffrey et al., 1976). While protein adducts cause little or no effects, DNA-adducts can give rise to mutations and subsequent carcinogenesis. This usually occurs by removal of the adducted DNA-base or misreplication of it.

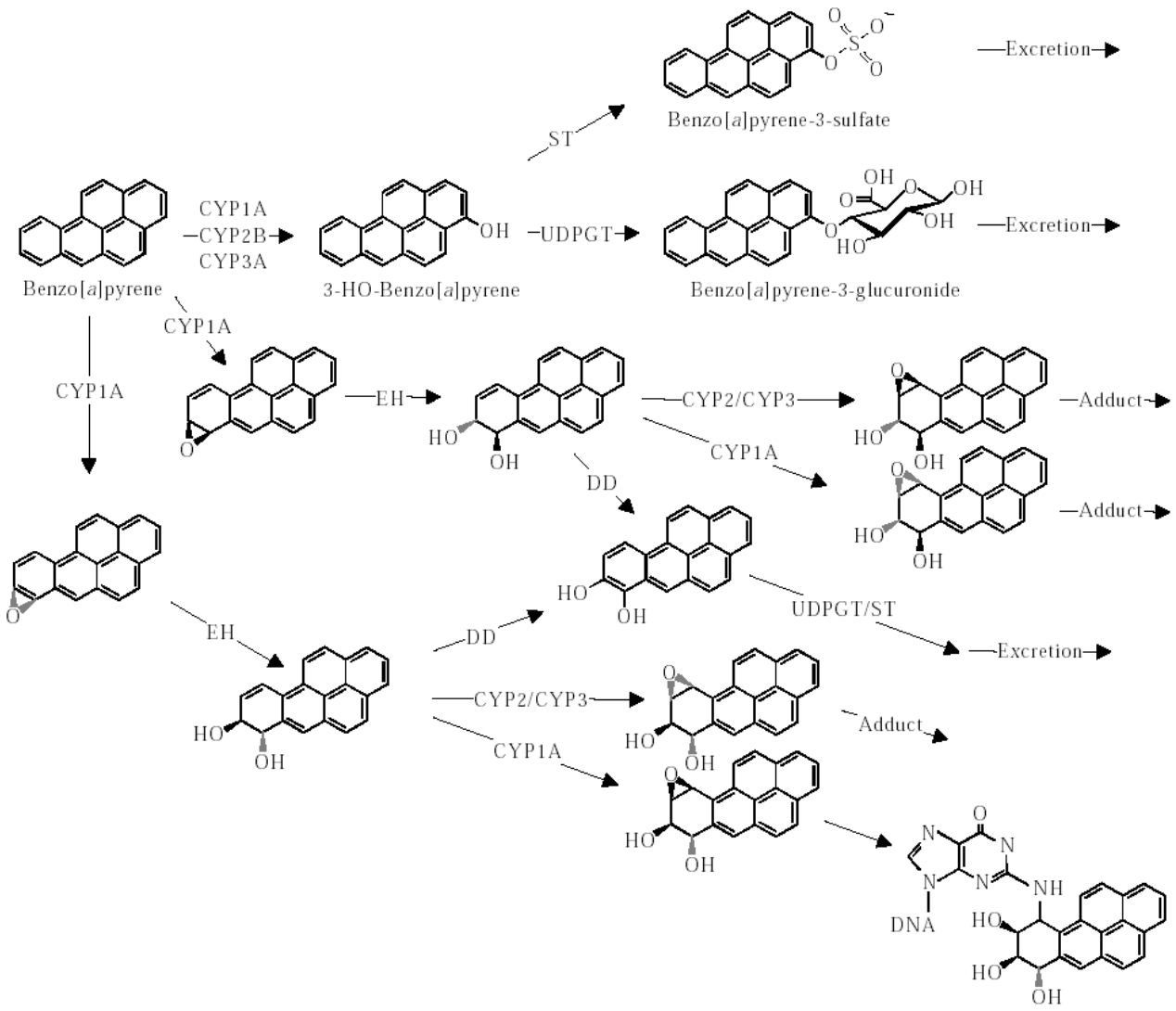

Figure 1.7 Biotransformation of benzo(a)pyrene as model PAH compound by cytochrome P450 enzymes (Source: Penning, 1993, Source: Stroomberg, 2002).

Endocrine disruption. The increasing concern of PAHs and many other persistent organic pollutants in the environment not only arises from their acute toxic or carcinogenic effects, but also from the increasing number of reports that they possess estrogenic characteristics, even at low concentrations. There is growing evidence that PAHs may also act as endocrine disruptors since several PAH metabolites, especially from the 4- and 5- ring PAHs, show affinity for the estrogen receptor (ER) α and β in a variety of test systems (Chaloupka et al., 1992; Tran et al., 1996; Clemons et al., 1998; Arcaro et al., 1999; Fertuck et al., 2001a). The explanation for this ER affinity lies in the structural similarity between hydroxy-PAHs and the natural hormone estradiol (Figure 1.8). Some hydroxy-PAHs may act similarly as estradiol by acting as a ligand to the human ER (pseudo-estrogens). They may also block the receptor causing failure of natural hormones to bind the receptor (anti-estrogens). This binding or blocking of PAH

metabolites is not only limited to the ER, but also takes place at the androgen receptor in which case androgenic effects are the result. This way, PAH metabolites may potentially interfere with estrogen signaling in important developmental and reproductive processes and lead to unnecessary promotion or inhibition of receptor depending gene transcription (Cooper and Kavlock, 1997). There is growing concern about human exposure to environmental pseudo-estrogens because they may result in reproductive malfunctions, teratogenic effects, decrease of sperm quality and even estrogen-related cancers.

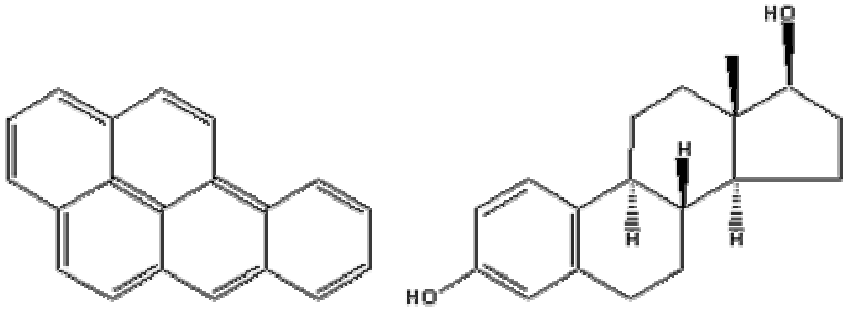

Figure 1.8 Molecular structure of b(a)p as model PAH compound and the natural estrogen 17β-estradiol

2.4.3 Microbial biotransformation activity

2.4.3.1 Types of microbial metabolism

The human large intestine harbours an incredibly diverse microbial ecosystem comprising 400-500 species at concentrations of 10^{11} microorganisms per gram gut content. For many years it was believed that the main purpose of the large intestine was the resorption of water and salt by the body and the facilitated disposal of waste materials. However, it has now become clear that the colon bacteria play a key role in many processes that are in direct relationship with our body. Numerous studies show the role of intestinal microorganisms in the synthesis of short chain fatty acids that provide energy to the colon epithelium (Cummings and Englyst, 1987), the stimulation of the gut immune system (Salminen et al., 1998), the synthesis of vitamins K and B (Conly and Stein, 1993) and the colonisation resistance against exogenous pathogens (Hopkins and Macfarlane, 2003).

Fermentation processes. Human colonic contents are a diverse mixture of bacteria, yeast, and other microorganisms, undigested or unabsorbed food, desquamated epithelial cells, bile, mucus, bacterial fermentation products, salts and water. The vast majority of the colon microbial population performs saccharolytic fermentation processes. Most simple sugars in the nutrition are already digested and

absorbed in the small intestine, but sugar molecules as raffinose, stachyose and lactulose and sugar alcohols such as sorbitol and xylitol reach the colon and become available to the resident microbiota. The main carbon and energy sources for the microbial population in the colon are resistant starches, plant cell wall polysaccharides and host mucopolysaccharides together with various oligosaccharides, proteins and peptides. The colon microbiota are able to ferment these substances to short chain fatty acids (SCFA), hydroxy- and dicarboxylic organic acids, H_2, CO_2, CH_4 and other end products (Macfarlane and Macfarlane, 1997). More in particular, SCFA production is important since these compounds are the main energy source for the colonocytes and influence colonic function by stimulating water and sodium absorption and modulating motility (Cherbut et al., 1997). It is estimated that from the 20-60 g d^{-1} of carbohydrates that are available in the colon (Cummings and Macfarlane, 1991), 300-500 mmol SCFA are daily produced with 95% being absorbed by the colon epithelium. Theoretical calculations show that up to 540 kcal d^{-1} might be obtained from carbohydrate fermentation, contributing up to 10% of the hosts daily energy requirements (Cummings, 1995). Additionally, butyrate also induces differentiation, stimulates apoptosis of cancerous cells *in vitro* and may thus inhibit cancer development (Scheppach et al., 1995; Reddy, 1999). Recently, Jan et al. (2002) demonstrated that also propionate induced colorectal carcinoma apoptosis. Moreover, propionate produced by the intestinal microbiota may contribute to plasma cholesterol lowering through inhibition of hepatic cholesterol synthesis (Chen et al., 1984; Wright et al., 1990; Demigné et al., 1995). Carbohydrate fermentation is by far the major force driving the activities of the colon microbiota. It is proportionally more important in the proximal part of the colon, this in contrast to the distal colon where amino acid fermentation is gaining importance, due to carbohydrate depletion.

The entire colon is an intensely proteolytic environment containing a complex mixture of bacterial and pancreatic peptidases. Proteolysis is an important ecological and physiological process in the colon because it provides nitrogen for growth by the different microbial groups in the colon. However, unlike carbohydrate metabolism, where the end-products are benign and may even be considered as beneficial to the host, many products of protein fermentation or putrefaction are toxic to the host. These include ammonia, indols, phenols and amines. A higher degree of proteolytic activity can therefore be associated to higher risks of colon cancer (Rowland, 1992). Ammonia concentrations may reach up to 40 mM in the colonic content which is well above concentrations of 10 mM which have already been described to alter the morphology and intermediary metabolism of intestinal cells, increase DNA synthesis and promote tumerogenesis (Ichikawa and Sakata, 1998). A higher proteolytic activity and lower

saccharolytic activity, especially in the distal part of the colon, would thus be more detrimental to human health.

Xenobiotic metabolism. Most resident colon microbiota typically perform fermentation processes, but several groups are also capable of transforming xenobiotic compounds (Macdonald et al., 1983; Ilett et al., 1990; Aura et al., 2002). The metabolic potential of the intestinal microbiota is considered to be enormous, rivalling that of the liver in the number of biochemical ractions and transformations in which it participates (Macfarlane and Macfarlane, 1997). Intestinal microbiota thus play an important role in the first-pass metabolism of compounds which are poorly or incompletely absorbed by the gut mucosa. A wide range of metabolic reactions can be catalyzed by a large diversity of microbial enzymes (Table 1.3). In contrast to the oxidative and conjugative reactions from the phase I and II enzymes in the enterocytes and hepatocytes, the bacterial metabolism is more reductive, hydrolytic and even of degradative nature with a great potential for both bioactivation as detoxification of xenobiotics (Ilett et al., 1990). Additionally, intestinal microbiota also interfere with the human biotransformation process through the enterohepatic circulation of xenobiotic compounds. Compounds that have been absorbed in the intestine and subsequently detoxified are usually conjugated with polar groups (glucuronic acid, glycine, sulfate, glutathion and taurine) in the liver prior to secretion with the bile (Ilett et al., 1990). Once released in the intestinal lumen, these conjugates may be hydrolyzed again by bacterial enzymes such as β-glucuronidase, sulfatase and other glycosidases. This would negate the detoxification cycle and delay the excretion of many exogenous compounds since the original compounds or phase I metabolites are more prone to intestinal absorption than their phase II conjugates. For example, guinae pig experiments showed that the hydrolysis of benzo(a)pyrene conjugates by intestinal microbiota resulted in *de novo* production of toxic benzo(a)pyrene intermediates which caused the formation of DNA adducts in the colon (Bowes and Renwick, 1986).

In summary, the colon epithelium may be exposed to potential toxicants from the non-digested nutrition and soil matrix, from the secretion of human phase II metabolites in the intestinal lumen, from the enzymatic activation of procarcinogens by the colon microbiota or from the direct production of toxic microbial compounds (McBain and Macfarlane, 1998).

Table 1.3 Metabolic potency of human gastrointestinal microbiota (after: Ilett et al., 1990).

Reactions	Enzyme	Bacterial species / origin of sample
Hydrolysis		
Glucuronides	β-glucuronidase	*E. coli*
Glycosides	β-glucosidase	*Enterococcus faecalis, Eubacterium rectale, Clostridium sphenoides*
Amides	Amide hydrolase	*E. coli, B. subtilis*
Esters	Deacetylase	*Enterococcus faecalis*
Sulfamates	Arylsulfotransferase	Clostridia, enterobacteria, enterococci
Reduction		
Azo-compounds	Azoreductase	Clostridia, lactobacillli
Unsaturated lacton	Unsat. glycoside hydrogenase	*Eubacterium lentum*
Aliphatic double bounds	Unsat. fatty acid hydrogenase	*Enterococcus faecalis*
Nitro-compounds	Nitroreductase	*E. coli*, bacteroides
N-oxides	N-oxide reductase	Human colon
S-oxides	Sulfoxide reductase	*E. coli*
Ketones	Hydrogenase	Rat caecum
Hydroxylamines	Nitroreductase	Rat GIT
Dehydroxylation		
Demethylaton	Demethylase	Enterococci, lactobacilli, clostridia
Deamination	Deaminase	Bacteroides, clostridia
Decarboxylation	Decarboxylase	*Enterococcus faecalis*
Dehydrogenation	Dehydrogenase	*Clostridium welchii*
Dehalogenation	Dehalogenase	*E. coli, Aerobacter aerogenes*
Synthetic reactions		
Esterification	Acetyltransferase	*E. coli*
N-nitrosation		*Enterococcus faecalis, E. coli*
Other reactions		
Oxidation	Oxidase	*E. coli, Enterococcus faecalis*
Isomerization	Isomerase	*Eubacterium rectale, clostridium sphenoides*
Fission aliphatic	Tryptophase	*E. coli, Bacillus alvei*
Fission ring	C-S lyase	Pig GIT, *Eubacerium aerofaciens*

2.4.3.2 Factors affecting microbial metabolism

The metabolic potency of the colon microbiota largely depends on the community composition. Basically, different microbial groups can be distinguished, each one capable of performing certain metabolic reactions such as carbohydrate breakdown, protein fermentation, endotoxin production and other metabolic processes. These microbial groups thus differ in their metabolic capabilities, which may often be the basis of classifying microbial groups according to their health-promoting or detrimental effects. This distinction was already conceptualized by Metchnikoff (1907) who argumented that some gut bacteria are beneficial to health, while others may be harmful. Gibson and Roberfroid (1995) reasoned that a classification as depicted in Figure 1.9 was conceivable. Yet, this can not be generalized since the beneficial and/or harmful effects of certain microbial groups largely depend on the conditions prevailing in the colon lumen or on the host himself.

Age. The age of a person is of major importance for the presence and absence of specific microbial groups throughout the gastrointestinal tract. Hence, as the microbial population changes, also the potency of performing metabolic reactions changes. The GI tract of the foetus in the mothers womb is sterile, but at birth, an infant's GI tract rapidly becomes colonized with organisms acquired from the mother and the local environment. In the first few days of life, enterobacteriaceae and enterococci are the predominant organisms in neonatal stool samples. These organisms create anaerobic conditions by oxygen depletion which allows the growth of bifidobacteria which become predominant after two weeks (Rotimi and Duerden, 1981) and which are stimulated by fresh human milk for breast-fed infants (Bullen et al., 1977). Formula-fed babies also have high bifidobacteria concentrations, but Enterobacteriaceae, Bacteroides spp. and clostridia remain at high levels. The difference in microbial composition between breast-fed and formula-fed infants may lead to early differences between individuals when investigating the metabolic potency of the intestinal microbiota. In some instances, the lack of xenobiotic metabolizing ability observed in infants is not due to absence of certain microbiota, but rather to the immaturity of the bacterial enzyme systems in the gut lumen. For example, the development of bacterial enzymes such as β-glucosidase and reductase can be reflected in the dependency with age of the metabolism of digoxin (Linday et al., 1987), cholesterol and methane production (Ilett et al., 1990).

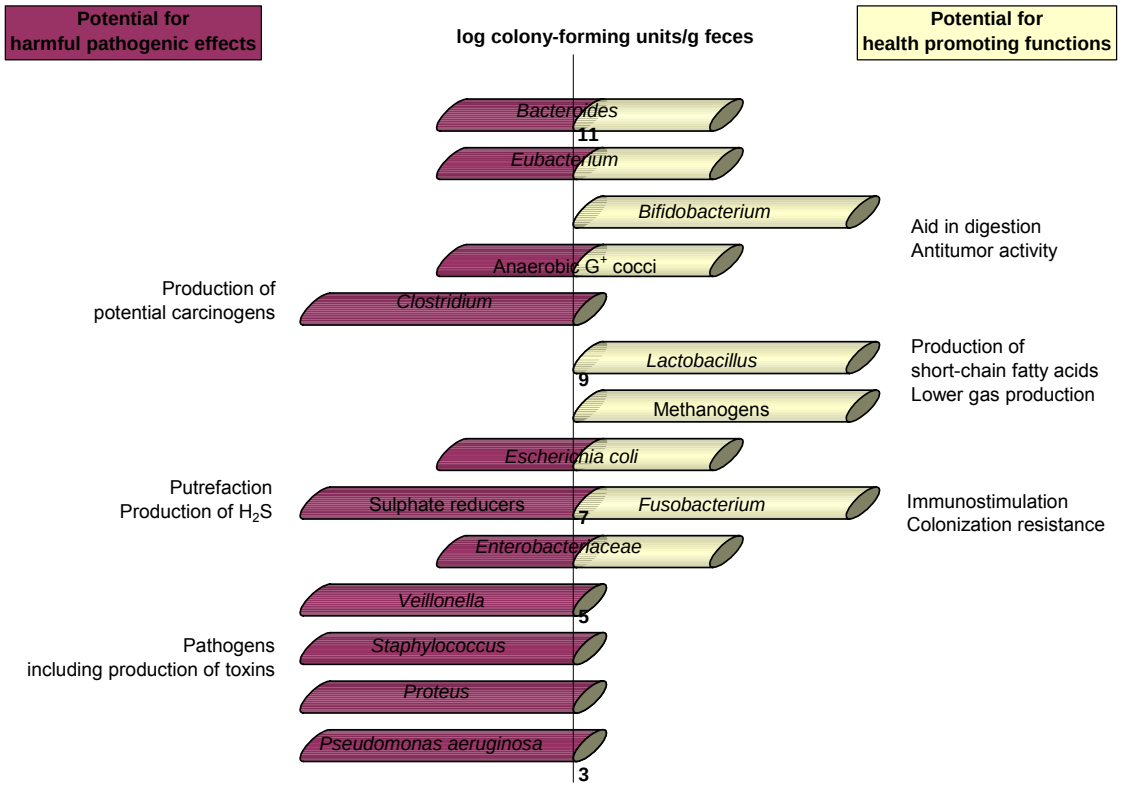

Figure 1.9 Dichotomy of the microbiota based on potentially toxic or beneficial properties (after: Gibson and Roberfroid, 1995; source: De Boever, 2000)

***Diet*.** The host's diet may significantly influence the composition of the intestinal microbial community and thus also its metabolic capabilities. It is known that the different diets consumed by various ethnic groups results in distinct microbial patterns in the intestine. An increased microbial colonization of the lower gut has been observed when dietary fibre content was decreased and refined carbohydrates and fat content were increased (Goldin, 1986) while a high-beef diet resulted in increased bacteroides populations in the human gut. Also the metabolic activity is influenced by dietary changes. A high cellulose and fibre content in the diet will decrease the intestinal transit time, increase the cecal size and the activity of cecal azoreductase, arylnitroreductase, β-glucosidase and β-glucuronidase in the rat (Rowland et al., 1983).

***Antibiotics and xenobiotics*.** (Ilett et al., 1990). Antibiotics largely influence the deconjugation enzymes that are responsible for the enterohepatic circulation of conjugated xenobiotic compounds in the body. In this way, the failure of oral contraceptives such as ethynylestradiol could be related to a concurrent antibiotic administration, lowering the enterohepatic recycling of the conjugated synthetic estrogen excreted in the bile. The too frequent use of antibiotic compounds since the

second world war as a treatment for bacteria-associated diseases has rapidly resulted in the emergence of antibiotic resistant strains. This involves not only changes in the cell membrane material, but also the metabolic potency of the resistant strain allowing them to inactivate antibiotic compounds.

In short, further research is needed to discover the metabolic capabilities of intestinal microbiota towards xenobiotic compounds and excreted phase II metabolites in the small intestine. Identifying those parameters that influence this capability and estimating the extent of the microbial metabolism may allow to achieve a more reliable assessment to what extent intestinal microorganisms contribute to the total risk that is posed by ingestion of environmental contaminants.

2.5 Oral bioavailability

2.5.1 Introduction

When native Indian communities in pre-Columbian South America were extracting curare from *Strychnos toxifera* plants, they understood quite well that this agent was harmless when ingested, but that it had a paralyzing effect when directly injected into their preys. Little did they know about the charged quaternary nitrogen atom in the alkaloid curare molecule which prevented transport of ingested curare across the intestinal epithelium thus avoiding the paralyzing effect in the organism. Similarly, the Egyptian Ebers Papyrus of 1550 BC reports on the ingestion of charcoal to take away the effects of poisons. Quite obviously, this ancient precursor of active carbon acted as an adsorbent towards toxic components, thus preventing uptake in the gastrointestinal tract and subsequent toxicity. These two examples illustrate that ancient civilizations had touched, without knowing, onto the concept of biological availability or ‘bioavailability’.

In essence, this term refers to observations that the concentration of a chemical in soil is not the actual concentration that the human body will experience. A complex mixture of physical, chemical and biological processes causes only a fraction of the measured chemical being available to impact the health of humans. These findings are part of other, more general reports which showed that hydrocarbons and other organic chemicals present in soil become less available due to interactions between the chemical and the soil and that this drop in availability lowers the risk of these chemicals to human (and ecological) receptors. With respect to oral exposure, differences in binding degrees of chemicals to the soil matrix result in different release

rates and thus intestinal absorption rates. The internal exposure of an ingested compound will therefore depend on the compound, the type of soil matrix or other factors such as fed or fasted state of the exposed subject (Linz and Nakles, 1997; NRC, 2003).

Measuring the bioavailable fraction of a compound has several consequences. Not only is it important to correctly assess the probable effects of contaminants towards human or ecological receptors. It may also influence the decisions that need to be made with regards to the determination of cleanup levels at contaminated sites (NRC, 2003). An adequate understanding and measurement of the bioavailability is therefore one of the key processes to make the outcome of risk assessment study more accurate and reliable.

2.5.2 Bioavailability definitions

General. Despite its critical importance to the risk assessment process, there is no clear consensus on how to define the term bioavailability. It has several definitions, depending on the scientific field in which it is used: biodegradation, plant uptake, human toxicology, ecotoxicology… (Table 1.4). It may represent the fraction of a chemical accessible to an organism for absorption, the rate at which a substance is absorbed into a living system or a measure of the potential to cause a toxic effect (NRC, 2003). Environmenal scientists usually consider bioavailability as being the accessibility of a solid-bound chemical to assimilation and possible toxicity (NRC, 2003), whereas pharmacologists and toxicologists consider it to be the fraction absorbed and able to reach the systemic circulation (Klaassen, 1986). Others consider a chemical as being bioavailable when it has crossed a biological membrane and becomes available at the site of biological activity.

Oral bioavailability. When handling issues of oral exposure to environmental contaminants, the term ‘oral bioavailability’ is used. In this respect, it was suggested that the oral bioavailable fraction is the amount of compound that is removed from soil through desorption processes in the intestinal lumen, subsequently followed by transport to the circulatory system. In this light, toxicologists also refer to the importance of the peak plasma concentration and the time to peak plasma concentration as important parameters in characterizing availability in the body. Measurements of these parameters may not always be useful in risk assessment since several compounds are also subjected to human metabolism and other physiological factors of importance, which influence the concentration of the parent compound in the plasma as such. Although all these definitions are very much related to each other,

they create confusion for environmental scientists and a significant level of uncertainty when measuring the bioavailable fraction of chemicals. Given the legal and regulatory implications of the bioavailability concept as part of the risk assessment framework, the term must be clearly understood.

Table 1.4 Definitions of bioavailability

Definition	Source
Generally used to describe the extent and rate of absorption for a xenobiotic which enters the systemic circulation in the unaltered (parent) form from the applied (exposure) site.	Hrudy et al., 1996
A concept that describes the ability of a chemical to interact with living organisms.	NEPI, 2000
The extent to which a substance can be absorbed by a living organism and can cause an adverse physiological or toxicological response	Battelle and Exponent, 2000
A term used to indicate the fractional extent to which a dose reaches its site of action or a biological fluid from which the drug has access to its site of action.	Wilkinson, 2001

The NRC bioavailability report (2003) aimed to bring some clarity by identifying different parameters that are all important prerequisites to the oral bioavailability of a compound. The bioavailability process, as depicted in Figure 1.10, incorporates exposure by release of soil-bound contaminant and subsequent transport, direct contact of a bound contaminant, uptake by passage through the intestinal membrane and incorporation into an organism.

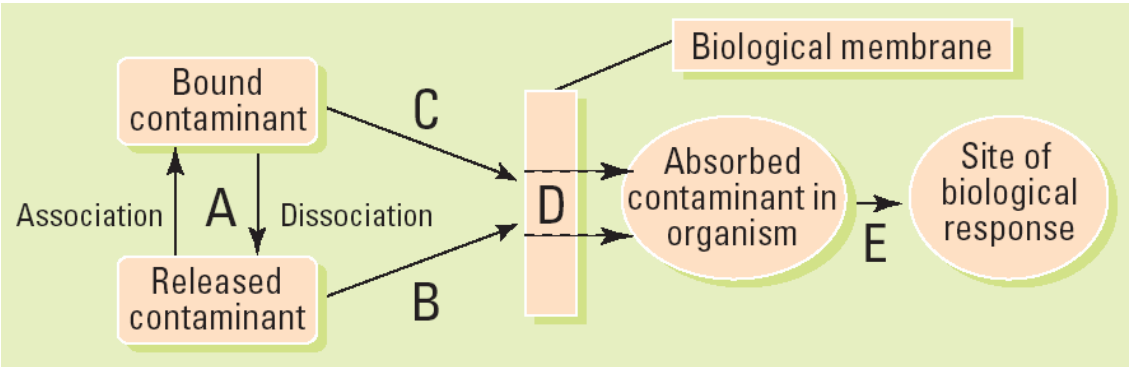

Figure 1.10 Bioavailability processes from soil contaminants by oral uptake (Source, NRC, 2003)

Following ingestion of a soil- or nutrition-bound contaminant, process A represents the contaminant release from its matrix and binding by other media in the intestinal suspension. It refers to the physicochemical and biological processes that influence the solubilization of a compound in the surrounding medium. Examples of parameters of influence are the octanol/water partition coefficients (K_{ow}) of the ingested contaminant as such, pH and redox values in the intestinal lumen, critical micelle concentration (CMC) for the mixed bile salt micelles, digestive enzymes or microbial metabolism. Process B involves the migration of a released contaminant to the membrane of an organism. Depending on the pKa value of the compound, this can be in an ionized or uncharged form or a released chemical can be integrated in mixed bile salt micelles which migrate to the intestinal epithelium. Process C involves the movement of contaminants that are still bound to the solid phase and that may interact from that state with the intestinal wall. Processes A, B and C all occur within the intestinal lumen. It is only from process D, that the movement of compounds from the intestinal lumen across a membrane into a living organism is envisaged. The exposure to both dissolved and solid-bound contaminants can therefore lead to chemical interaction with the membrane of an organism and subsequent uptake. Process E in Figure 1.10 refers to the subsequent paths of a chemical following absorption. It may be biotransformed, bioactivated, transported and exert a toxic effect at the site of biological response. The various definitions of the term “oral bioavailability” often disagree whether or not process E should be incorporated or not. The reasoning behind this is that chemicals which are detoxified during the biotransformation process, may be excreted again from the body via the bile or urine. Oomen et al. (2002) for example consider the biotransformation processes in the liver to be part of the oral bioavailability process. A formula incorporating different parameters in the equation thus allows to calculate the bioavailable fraction:

$$F = F_{BAcc} * F_{Abs} * F_{Met}$$

with F_{BAcc} being the bioaccessible fraction (the fraction which is released in the intestinal lumen and at maximum available for uptake), F_{Abs} being the fraction transported across the intestinal membrane and F_M being the fraction which passes the liver without being metabolized. For instance, the bioavailability of soil-bound lead upon soil ingestion would be 20% if the released fraction from the soil (F_{BAcc}) is 40%, the fraction absorbed (F_{Abs}) 50% and the fraction passing the liver 100%.

This equation includes process E from Figure 1.10, in correspondence with the definition by Hrudy et al. (1996) who focused on the parent compound itself. This is in agreement with medical and toxicological literature, where the parameters F_A and F_H are taken together under the term ‘absorption’. It pertains to absorption into the

systemic blood stream, i.e.: absorption includes both intestinal absorption and the process of first-pass metabolism.

According to Semple et al. (2004), this interpretation may however present a problem to risk assessers since it does not include that absorbed contaminants may be biotransformed to bioactive compounds which may reside in the body. This way, an underestimation of the actual biological availability of a contaminant is obtained, since the metabolites in the body are not considered. In this light, it is better to have a more conservative oral bioavailability definition, which does not depend on the degree of biotransformation activity. Semple et al. (2004) therefore avoid this approach and define bioavailability as “that which is freely available to cross an organism’s cellular membrane from the medium the organism inhabits at a given time.” Processes which do not involve the transfer of a contaminant between its matrix and the organism – storage, assimilation, transformation or degradation – are therefore not part of the term bioavailability. Translated to human contaminant exposure by oral uptake, this means that biotransformation processes are not considered and that from Figure 1.10, only processes A through D are taken up in the assessment. Despite these conflicting interpretations of the term ‘bioavailability’, it is generally agreed that the entire process of a compound to become bioavailable encompasses several processes, including the release from the soil matrix, uptake across the intestinal epithelium, first-pass effect and distribution to specific organs or cells (NRC, 2003).

Calculations. Developing a transparant procedure for performing risk assessment studies therefore requires some consensus in how to define oral bioavailability and to clearly envisage what fraction of an ingested compound will be measured. To aid in the elucidation of the bioavailability process and to allow a pragmatic application of the term bioavailability in risk assessment studies, two measures of oral bioavailability can be distinguished, especially for HHRA. Absolute bioavailability is the fraction of a compound which is ingested, actually absorbed and reaches the systemic circulation (Hrudy et al., 1996). Absolute bioavailability (BA) can be defined as the ratio of an internal dose (D_{int}) to an external dose (D_{ext}):

$$BA = \frac{D_{\text{int}}}{D_{ext}} \times 100$$

In other words, the absolute bioavailability indicates the relationship between the external dose and internal dose of a contaminant to which an organism is exposed (Figure 1.11).

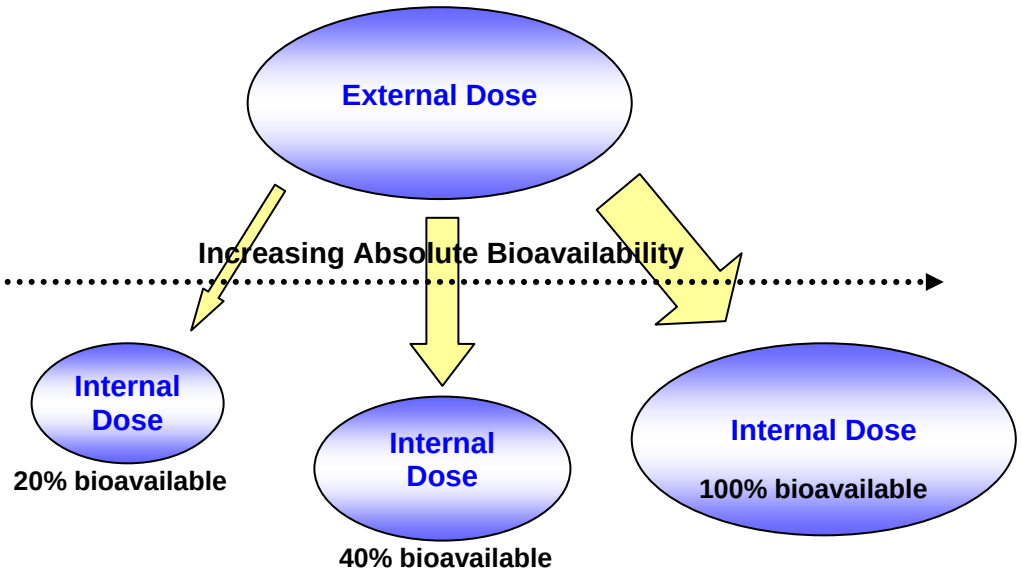

Figure 1.11 Influence of bioavailability onto the internal dose of a chemical

For studies of absolute bioavailability, the absorbed dose is often determined by measuring the concentration of the compound in blood over time or by measuring the mass of the compound in such excreta as urine, feces, or exhaled air (Batelle and Exponent, 2000). However, most human toxicity data used in risk assessments are based on laboratory animal studies in which the chemical is delivered to the test animal in a pure form or as part of a solid or liquid diet. Since the bioavailability of an ingested chemical in these toxicity studies is different than in soil, there is a need to develop correction factors. The absolute bioavailability is therefore not often used in HHRA. Additional tests for estimating contaminant bioavailability, especially for hydrophobic contaminants such as PCB, TCDD or pesticides, include measuring the contaminant concentration in the fat tissue of an organism.

Relative bioavailability (RBA) is a measure of the extent of absorption among two or more forms of the same chemical (e.g., lead carbonate vs. lead acetate), different vehicles (e.g., food, soil, and/or water), or different doses (Batelle and Exponent, 2000). Matrix effects substantially influence the bioavailability of a soil-bound or nutrition-bound contaminant, compared to the medium that was used to test the toxicity of that same contaminant. Relative bioavailability can be calculated as:

$$RBA = \frac{F_s}{F_{sm}}$$

where F_s is the fraction of the chemical absorbed from soil under circumstances of environmental exposure and F_{sm} is the fraction of the chemical absorbed from the study matrix which is used in toxicity studies. Basically this means that the aspect of

oral bioavailability is incorporated in this equation, since it takes into account that ingested chemicals may differ in absorption, depending on the matrix in which they were ingested. If the absorption from soil is found or assumed to be the same as absorption in the toxicity study, then the RBA is 1.0 (or 100%). This does not necessarily mean that the absorption as such is complete, since the absorption of a compound in the toxicity study may also be uncomplete (thus in theory the RBA can even be higher than 100%), depending on the test chemical itself or on the physiological conditions of the test organism. The relative bioavailability factor thus accounts for the difference in oral bioavailability caused by the matrix of ingestion (NRC, 2003). If there is no difference between the chemical form or test matrix that was used in the toxicity study and that which is of interest in the risk assessment of a contaminated soil, there is no need to determine the RBA.

2.5.3 Use of bioavailability in risk assessment

The previous discussion on what bioavailability means and what types of measures can be distinguished, is of significant importance to the risk assessment process for oral exposure to soil bound contaminants. It suggests that greater attention to this biological availability or 'bioavailability' of chemicals is needed to realistically evaluate their potential risk. Hence, current risk assessment procedures not only compare contaminant concentrations from the soil with risk-based screening levels from toxicity studies, which represent a conservative estimate of risk. They also incorporate the input of site-specific parameters, including bioavailability, thus decreasing the uncertainty levels and making a more accurate site-specific risk assessment possible.

Incorporation of bioavailability into risk assessment happens at all four levels of the risk assessment process: risk identification, dose-response assessment, exposure assessment and risk characterization. The risk identification step at a contaminated site allows to determine the different risk groups and the pathways of how soil contaminants may end up in the human gastrointestinal tract. For example, ingestion of fine inhaled soil particles by construction workers or ingestion of larger soil particulates will clearly affect the bioavailability of the soil contaminants and also the selection of a specific method to investigate the actual exposure.

As discussed above, the dose-response assessment entails the generation of toxicity values that are based on several biological assays (*in vivo* or *in vitro*) in which the relationship between the dose of a chemical and its toxic effects are evaluated and which allow an extrapolation to the field situation. Although bioassays are valuable tools in the characterization of toxic action of chemicals and in the understanding of

associated toxicity, they do not consider all the processes in the body or other environmental factors that influence toxicity (Fent, 2003). Most toxicity values are therefore based on applied doses, rather than the doses to which the human body is really exposed. This implies that the toxicity value is still a function of the rate and extent of absorption that occurs in the study. In other words, it is dependent on bioavailability processes that are not inherently part of those processes that make up the actual toxicity of a compound (Figure 1.12). Hence, working with toxicity values and performing reliable risk assessment studies implies a proper understanding of those processes that influence bioavailability, such as the interaction of freely dissolved contaminants with dissolved or particulate organic matter in the intestinal lumen.

During the exposure assessment, knowledge of the bioavailability of a contaminant in soil is necessary to accurately estimate the fraction of the exposure dose of the contaminant that has the potential to impact human health. The soil ingestion equation as defined in part 1.2 incorporates bioavailability data. It can either be used to modify the chemical concentration in the soil (Cs) by expressing it as the concentration of the bioavailable fraction of contaminant rather than the chemical concentration, or it can be used in the relative absorption factor. Since most risk assessment studies currently assume that the absorption of soil-bound contaminants is 100%, there may be a large overstimation of the actual risk posed by that contaminant. Further research investigating those processes that precede bioavailability is therefore warranted.

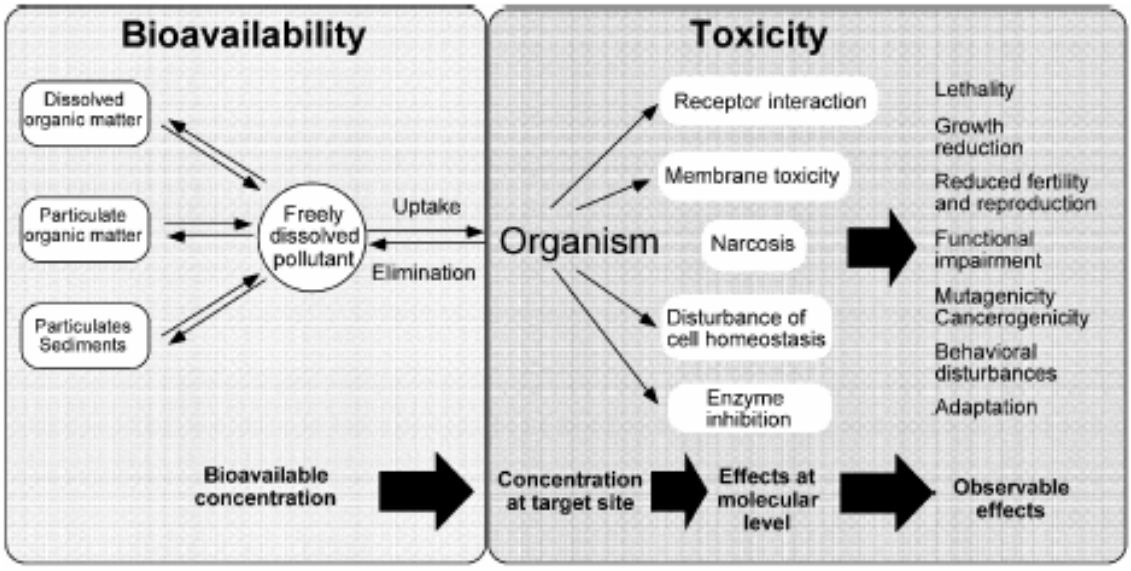

Figure 1.12 Ecotoxicological effects are dependent on the bioavailable fraction of pollutants, and concentrations at the target sites induce molecular effects that propagate to a variety of toxic manifestations in organisms. Adapted from Escher et al. (1997).

The final step in the risk assessment process determines whether the risk from soil-bound contaminants when ingested, exceeds acceptable levels. From the dose-response and exposure assessment, it can be concluded that the bioavailability process will impact the internal exposed dose and also the subsequent risk. The risk characterization step which relies on the PEC/PNEC ratio, therefore also relies on accurate bioavailability measurements. More specifically, bioavailability is inversely related to risk-based cleanup levels from soil (Figure 1.13). The regulatory consequences of the bioavailability concept are thus considerable.

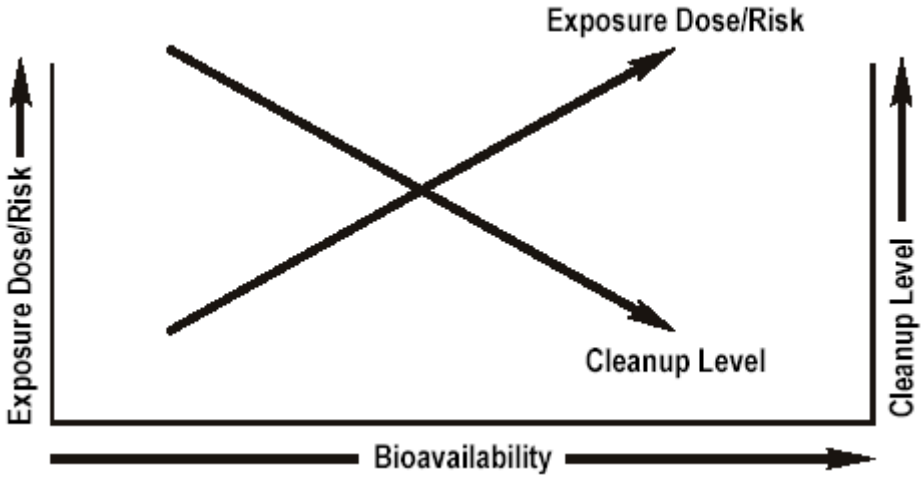

Figure 1.13 Relationship between bioavailability and risk assessment endpoints (Batelle and Exponent, 2000).

2.5.4 Bioaccessibility – a novel concept in HHRA

As discussed above, the measurement of bioavailable fractions of ingested contaminants is crucial for a successful HHRA. *In vivo* experiments allow a comprehensive study of the fraction of a chemical reaching the blood compartment and its toxicity. Yet, those processes that preclude intestinal absorption are a black box to these experiments. Several parameters play a crucial role and at this point, it is important to introduce the term bioaccessibility which is basically a compilation of processes A, B and C in Figure 1.10 describing a fraction of the ingested contaminant which comes into consideration for intestinal absorption. This differentiation of bioaccessibility from bioavailability corresponds to the definition from Hamel et al. (1998) who state that “The bioaccessibility is defined as the maximal amount of contaminant that is soluble in a synthetic gastrointestinal fluid and therefore potentially available for uptake across the intestinal lumen.” Other researchers have also

identified the fraction of the contaminant that is mobilized from soil into the digestive juice chyme as the bioaccessible fraction (Ruby et al., 1996; Ruby et al., 1999; Oomen et al., 2002). This fraction is considered to represent the maximum amount of contaminant available for intestinal absorption. Bioaccessible contaminants can subsequently be absorbed, that is, transported across the intestinal wall, biotransformed by the liver and transferred to the blood or lymph. Assessment of the bioaccessibility of an ingested soil-bound chemical can be very useful for a final estimate of the oral bioavailability. Bioaccessibility is thus an important parameter to integrate in the exposure assessment step.

A series of compartments are involved in human uptake of ingested soil contaminants. The overall pathway leads the soil with contaminants from the mechanical grinding in the mouth through a series of chemical and microbiological processes to partial dissolution through the entire gastrointestinal tract (bioaccessibility processes). Most of the dissolution processes are completed before the material is leaving the small intestine, and it is generally accepted that most of the uptake takes place in the small intestine. The physicochemical conditions in the intestinal lumen may depend on the physiological state of the host (fasting or eating), the age, the contaminated matrix which is ingested… All these processes and parameters influence the bioaccessibility of ingested contaminants and therefore, also their bioavailability (Oomen et al., 2002). The most important functions and parameters of influence of the bioaccessibility process occur in the mouth, stomach and small intestine. The mouth primarily has a grinding function, decreasing the size of the ingested matrix, and the enzymatic cleavage of starch by amylase. The residence time varies from seconds to minutes and the degree of grinding is of large influence for the dissolution of a contaminant from its matrix.

The specific effects of the stomach conditions towards soil-bound chemicals is mainly oriented towards dissolution and release of chemicals in the gastric suspension. This is established by pH values that may go as low as 1, but that may also be as high as 4 under fed conditions. Depending on the fed or fasted state, the residence time in the stomach may vary from 3 hours to only 10 minutes. The contaminant dissolution functions in the small intestine are more variable and complex. The pH in the small intestine varies from 4.5 to 7 whereas the residence time is usually between 3 and 10 hours. A large number of dissolution and complexation processes in the small intestinal suspension may further release contaminants from soil, but on the other hand, several complexation reactions may also occur as for example with metals that are precipitated at higher pH values or with phosphate. Cationic metals and hydrophobic contaminants are more easily solubilized by the complexation with bile

salts which increases the bioaccessible fraction and thus their chance of being absorbed across the intestinal epithelium. Also, lipids and other soluble organic matter in the intestinal suspension can add to the carrier effect of the bile.

Clearly, the complexity of processes occuring in the gastrointestinal tract are of large influence to the bioavailability of an ingested soil-bound contaminant. For the relative bioavailability process, bioaccessibility would thus represent a very important rate limiting step, next to intestinal absorption. The bioaccessibility process is very sensitive to the different luminal factors from the gastrointestinal tract, whereas intestinal absorption of bioaccessible compounds is less sensitive to luminal processes and biotransformation of absorbed contaminants would be insensitive. Assessment of the relative bioavailability of a chemical would thus rely on the measurement of the bioaccessibility and the intestinal absorption as the two rate limiting steps. If the bioaccessibility process is rate limiting (i.e. if dissolution is slower than absorption), then bioaccessibility will determine the relative bioavailability. If the absorption process is rate limiting (i.e. absorption of dissolved contaminants is to slow to be completed before transit), the intestinal absorption will dominate the relative bioavailability.

2.5.5 Problems with bioaccessibility

The term bioaccessibility for environmental contamination was introduced by Ruby et al. (1993) who investigated the relative solubility of ingested lead (Pb) from different mine wastes in the gastrointestinal tract. Since then, it has been used for other contaminants such as heavy metals, lindane, PCBs, PCDD/Fs, PAHs, radionuclides (Hack and Selenka, 1996; Davis et al., 1997; Hamel et al., 1999; Oomen et al., 2000; Ellickson et al., 2002; Van de Wiele et al., 2004a) and also for nutritional constituents such as isoflavones, folic acid, and carotene (Arkbåge et al., 2003, Vuong et al., 2003; Walsch et al., 2003). Bioaccessibility measurements for site-specific risk assessment studies are significantly gaining importance since they offer an efficient, cheap and high-throughput alternative to the time-consuming and expensive *in vivo* methods that are often used.

However, there is one big flaw in the current use of the bioaccessibility concept: almost every research group that applies it, uses another method to measure the bioaccessible fraction in the intestinal lumen. The two causes are that 1) the *in vitro* models all differ in complexity and digestion parameters (different enzyme concentrations, pH values and residence times) and 2) different methods for separating bioaccessible from non-bioaccessible contaminants are used. There is currently no consensus at all what seperation process is best. Depending on the used

in vitro method or on the compound under study, researchers have used 0.45 µm filtration (Ruby et al., 1996), 3000×*g* centrifugation (Oomen et al., 2001; Ruby et al., 2002), 7000×*g* centrifugation (Hack and Selenka, 1996), a combination of 1100×*g* centrifugation with 0.45 µm filtration (Holman et al. 2002), 10 kDa ultrafiltration (Minekus et al., 1995)… Obviously, using different methods to measure the bioaccessible fraction will lead to a different outcome when soils are tested.

Although several *in vitro* methods have been validated against *in vivo* data (Minekus et al., 1995; Hack and Selenka, 1996; Rodriguez and Basta, 1999), this has only been done under well-defined circumstances and it can not always be extended to other site-specific situations. Obviously, if all methods claim to give an estimate of bioaccessibility and if all methods are substantially different, different correction factors that relate bioaccessibility to oral bioavailability need to be implemented, which unnecessarily adds to the uncertainty of the risk assessment. Additionally, if bioaccessibility values for one single contaminated soil change with the used *in vitro* method, the risk assessment process itself is totally unreliable.

For this reason, the BioAccesibility Research Group of Europe (BARGE) was established in 2000 which assembles several scientific and governmental institutes from Europe (Figure 1.14). The actively involved partners are affiliated with the British Geology Survey (UK), University of Nottingham (UK), TNO Nutrition and Food Research (The Netherlands), The National Institute for Public Health and the Environment (RIVM, The Netherlands), DHI Water and Environment (Denmark), The Flemish Institute for Technological Research (VITO, Belgium) and Ghent University (Belgium). American and Canadian partners take part of the discussions and are affiliated with Ohio State University, Oak Ridge National Laboratories and Jacques Whitford Environment.

Figure 1.14 Logo of the BioAccessibility Research Group Europe.

The goal of BARGE is to compare and evaluate the many models and systems that have been developed over the years to estimate bioaccessibility and contaminant exposure. The ultimate goal is to develop a methodology which indicates what the default digestion parameter values are for different risk groups and which parameters need to be changed to specifically use *in vitro* models for site specific risk assessment. From the BARGE viewpoint, it is assumed that more realistic and defensible bioaccessibility factors can be obtained which can then be used for risk assessment and policy making. In a first research initiative, Oomen et al. (2002) showed that indeed the variability among *in vitro* digestion models is extremely high and that this is also translated in the outcome of bioaccessibility measurements. Five different *in vitro* models of the human gastrointestinal tract were used to measure lead, arsenic and cadmium bioaccessibility from different contaminated soils. The *in vitro* digestion models produced a wide range of bioaccessibility values for three contaminated soils (Table 1.5).

Table 1.5 Bioaccessibility values (%) for arsenic, cadmium and lead in Montana 2711 soil as obtained from 5 *in vitro* digestion models of the human GI tract.

	Arsenic	Cadmium	Lead
SBET (BGS)	59	99	90
RIVM method	50	79	68
DIN (Bochum University)	59	40	11
SHIME (Ghent University)	10	6	3
TIM (TNO-Nutrition)	50	58	17

Clearly, these numbers reveal nothing more than that there is a large variability in the data set due to differences between the model digestion parameters. Comparison of specific parameters however revealed that key parameters in the bioaccessibility process were the low stomach pH and the presence of food particles, both positively influencing bioaccessibility of soil-bound heavy metals. The acidic environment in the stomach mobilizes more easily metals thus increasing the released fraction, while food particles provide complexation niches in the intestinal liquid matrix, thus driving the contaminant equilibrium between soil and chyme towards desorption from the soil matrix. It was concluded that organic matter from food particles enhance mobilization and thus the bioaccessible fraction. Chapter 2 of this dissertation will study these *in vitro* methods in more detail.

3. Studying the human GI tract

3.1 In vivo experiments

The main advantage of *in vivo* models is that the complexity of physiological parameters is integrated in the experiments. Investigating desorption and complexation processes in the gut of resp. oligochaetes, other invertebrates, fish, rodents and other mammals has proven to be useful as relevant tests of oral bioavailability (Gomot-de Vaufleury and Pihan, 2002; Koganti et al., 1998; Loonen et al., 1997; Oste et al., 2001; Yan and Wang, 2002; Willett et al., 2001). Additionally, metabolic studies using conventional and germ-free or gnotobiotic animals may be used to investigate the metabolism by gastrointestinal microbiota (Hirayama et al., 2000). In general, drug absorption in animals is believed to be a good predictor of absorption in humans. Animals integrate all the biological factors that may affect drug absorption and metabolism. Unlike *in vitro* systems, in which a correlation to *in vivo* data must be established, this step is unnecessary when animals are used (Hidalgo, 2001). An additional advantage of using animals for oral bioavailability and microbial metabolism studies is that the used species in these studies can be same as those used in pharmacological and toxicological studies.

Disadvantages to *in vivo* studies are the relatively large amount of material that is needed, the complexity of analytical methods for the analysis of blood and tissues and the time-consuming and labor-intensive nature of the experiments. Additionally, *in vivo* experiments provide little mechanistic information on the processes that are important prerequisites to oral bioavailability. It is impossible to separate the variables involved in the process of absorption, thus making identification of individual rate-limiting factors impossible. Hence, contaminant release and complexation processes that influence the bioaccessible fraction of a compound inside the GI tract are unknown mechanisms in such studies. Moreover, given the discrepancies between the physiology of humans and other organisms, as well as the low reproducibility and ethical constraints associated with such studies, a more efficient alternative is desirable (Ruby et al., 1999). *In vitro* models from the human GI tract have therefore been developed.

3.2 *In vitro* experiments

In vitro digestion experiments are specifically designed to aid in the exposure assessment step of the risk management process. Although the outcome of such studies may significantly contribute in the interpretation of bioavailability data, the value of *in vitro* digestion experiments may not be overestimated since it merely deals with a physicochemical approach. In contrast to *in vivo* tests, *in vitro* experiments should focus solely on bioaccessibility measurements because this step in oral bioavailability is assumed to represent the step that is most sensitive to the ingestion matrix. In addition, an *in vitro* model facilitates a systematic investigation of many variables (Oomen et al., 2003) thus focusing on all processes and parameters that influence the dissolution of a compound from its matrix and its availability for intestinal absorption. *In vitro* digestion models that mimic the human GI tract can elucidate these phenomena. These models, based on the physiology of humans, are generally more simple, less time-consuming and especially more reproducible than animal tests. Tests that simulate stomach and small intestinal conditions (Hack and Selenka, 1996; Ruby et al., 1996; Jin et al., 1999; Oomen et al., 2000) and intestinal transport through Caco-2 tissue cultures have been described before (Oomen et al. 2001) and could provide a rapid and inexpensive method for developing more accurate exposure estimates for use in human health risk assessments. Specifically for the risk assessment of contaminants ingested with soil, Oomen et al. (2003) define a couple of requirements that an appropriate *in vitro* method should fulfill:

- Representation of human physiology, and more specifically, physiology of children, because soil ingestion is an important route of exposure for children due to frequent hand-to-mouth behavior (Duggan and Inskip 1985; Davis and Waller 1990; Calabrese et al. 1997a).
- Representation of a worst-case situation, but this should be as realistic as possible. This situation is compound-dependent.

Several *in vitro* methods are sequential extractions with two distinct extraction steps: a gastric-phase extraction that simulates the acidic biochemical stomach environment and a subsequent intestinal-phase extraction that simulates the biochemical environment of the small intestine (Schröder et al. 2003). The bioaccessible fraction of the soil contaminants that are subjected to these two steps, is then related to the relative bioavailable fraction by validation with animal models. This approach of mimicking the gastrointestinal conditions in a two-phase extraction test has been used to estimate the bioaccessibility of lead (Ruby et al., 1996; Oomen et al., 2001, 2003), cadmium (Hamel et al., 1998), arsenic (Rodriguez and Basta., 1999) and

also some organic contaminants such as lindane (Oomen et al., 2000), PCB (Hack and Selenka, 1996) and PAHs (Van de Wiele et al., 2004a).

In vitro models can be split up in static and dynamic models. Most models are static gastrointestinal models, which simulate transit through the human digestive tract by sequential exposure of the soil to simulated mouth, gastric, and small intestinal conditions. In the simplest approach, the *in vitro* stomach model, mobilization of the contaminants from soil under gastric pH conditions is simulated. The best known example of an *in vitro* stomach model is the SBET method or Simple Bioaccessibility Extraction Test which simulates the mobilization of contaminants in the acid conditions of the stomach. An intestinal compartment is not employed. This model is adapted from a model described by Ruby et al. (1993), which was initially developed for measuring lead bioaccessibility but which has also been used for other metals (Oomen et al., 2002). This model has been validated to the *in vivo* situation using swine studies (Ruby et al., 1999). Although the SBET method has proven its applicability in several studies, its major disadvantage is that it does not include a small intestinal step. Although correction factors are used to relate the SBET experimental data to relative bioavailability estimates, the uncertainty is fairly high and the actual release complexation process of contaminants are not elucidated. Several static models have therefore been developed to also include the small intestinal step to arrive to more realistic conditions for the gastrointestinal lumen. The PBET method stands for the Physiologically Based Extraction Step and is used by Ruby et al. (1999) and the BGS institute (UK) for the bioaccessibility assessment of lead and arsenic. The German method E DIN 19738 has its origin in the *in vitro* digestion models by Rotard et al. (1995) and Hack and Selenka (1996). It is a static *in vitro* gastrointestinal model using synthetic digestive juices. Because it is assumed that saliva has only a negligible effect on the level of mobilization of contaminants from soil, only synthetic gastric and synthetic intestinal juices are used. Whole milk powder (50 g/L) may be added to the test system to simulate the influence of food on the mobilization of contaminants. The National Institute of Public Health and the Environment (RIVM) in the Netherlands has also developed a static *in vitro* model which resembles that of Germany. However, a saliva step is also introduced into this model to simulate the biochemical conditions from the mouth.

In contrast to these static models, dynamic gastrointestinal models mimic the gradual transit of ingested compounds through the digestive tract and may mimic more aspects of the human physiology. Major disadvantages are the complexity and cost of such models and also the fact that only a limited number of samples can be tested at

the same time. A first example of a dynamic model for the human gastrointestinal tract is the TIM model (TNO Intestinal Model) that simulates the transit through the gastrointestinal tract, the gastric and intestinal pH profiles, and the secretion of digestive juice over time (Minekus et al., 1995). This model has been validated by comparing the dissolution profile of drugs in vivo and in vitro, and with food components. The experiments with soil are performed while reproducing conditions that occur during digestion of a semiliquid meal.

A second example of dynamic models is the SHIME (Simulator of the Human Intestinal Microbial Ecosystem) which effectively models the human intestinal tract (Molly et al., 1993; 1994). It is an automated and robust multi-stage reactor that is amenable to bioavailability estimates. This computer-controlled dynamic model consists of five compartments, each compartment vessel simulating a different part of the GI tract from the stomach and small intestine to the proximal and distal part of the colon. The SHIME reactor differentiates itself from other in vitro intestinal models, because it comprises the whole GI tract taking into account the enzymatic processes in stomach and duodenum and the different characteristics of the microbiota along the colon reactors. Models such as these allow to investigate the biochemical and physical processes, contributing to contaminant release from soil matrices inside the human gastrointestinal tract.

In vitro models of the gastrointestinal tract are also specifically suitable when research has to be carried out on the intestinal microbiota. Since the proximal colon is unaccessible to routine investigations, microbiological research on the GI tract is usually carried out by isolating microorganisms from fecal matter and growing them in batch cultures or one-compartment chemostats. The most simple experimental setup for studying the metabolism of intestinal microbiota is an anaerobe batch reactor. In these fermentor vessels suspensions of fecal material are brought in a buffered growth medium and incubated under anaerobic conditions. This method is very userfriendly given its simplicity. Yet, it is only useful for limited periods of time since environmental parameters such as pH, redox potential, available nutrients and microbial population dynamics constantly change. In order to maintain the inoculated intestinal microbiota over a longer timeframe, semi-continuous fermentor vessels were developed where the intermittent supplementation of nutritional medium and the removal of microbial suspension could be simulated (Miller and Wolin, 1981; Manning et al., 1987). The latter systems typically make use of one single fermentor. The colon is however a very heterogeneous region with clear differences in substrate availability, fermentation activity, microbial composition and several environmental conditions. This makes it

impossible to simulate a representative culture of colon microbiota in one compartment (Macfarlane et al., 1998). Several multi-compartment reactors were developed to simulate the different conditions of the colon lumen (Veilleux and Rowland, 1981; Macfarlane et al., 1989; Miller and Wolin, 1981; Molly et al., 1993; Minekus et al., 1999). In light of the research that was conducted for this PhD, the TIM2 model from TNO Nutrition and the SHIME developed by LabMET are the most important examples.

In vitro digestion models are likely to become increasingly important in risk assessment of polluted soils, especially for determining the urgency of remediation (Oomen et al., 2002). At present there is no standard method to estimate bioaccessibility. Many digestion models exist with various experimental designs. The different models rarely result in similar bioaccessibility values and it needs further investigation to identify the key parameters that influence the bioaccessibility of a compound in these models. Future research should aim at narrowing the window of uncertainty and arrive to a standard procedure of how to use the different models to assess bioaccessibility.

4. Objectives

Ingestion is one of the most important exposure routes by which contaminants may enter the human body. Assessing the risks that are associated with this exposure necessitates an adequate understanding of the different steps that are involved from the ingestion of a contaminant to its bioavailability and eventual toxicity. In this PhD research two aspects were studied in more detail: **bioaccessibility** and **microbial biotransformation**. The reason for this is that literature study indicated that these parameters are not fully understood or investigated when estimating health risks from ingested soil contaminants.

Firstly, the use of *in vivo* experiments using rats, mice, guinae-pigs or primates as the most relevant approach to assess contaminant bioavailability does not allow a complete elucidation of those processes that are important processes determining the bioavailable fraction. Luminal processes towards ingested contaminants remain a ‘black box’ to these types of *in vivo* experiments. Secondly, specific toxicokinetic and pharmacological studies allow to investigate the processes to which chemicals are subjected once transported across the intestinal wall. Yet, the non-absorbed chemicals may still become available to colon microorganisms that are capable of metabolizing

chemical compounds, not only nutritional but also xenobiotic. Except for some pharmaceutical compounds, not much information is available about this microbial metabolic potency. These two issues will be addressed in this PhD research in the following outline.

Chapter 2 will first describe different *in vitro* methods of the human gastrointestinal tract that have been used to assess the bioaccessible fraction of soil-borne lead. The outcome of the different methods will be compared and possible differences will be explained by parameters that distinguish one model from the other. Additionally, a brief comparison will be made with *in vivo* data from a study in which the bioavailability of lead from that same soil matrix was measured with human volunteers. In contrast to this chapter that deals with lead, the other research chapter all investigated bioaccessibility or microbial biotransformation of PAHs. The reason for this is that the comparison study from **Chapter 2** was conducted within the BARGE consortium. Due to the high complexity of the different bioavailability and bioaccessibility processes, the BARGE consortium has decided to initially investigate the behavior of soil-borne heavy metals in the gut. Studying organic contaminants such as PAHs currently causes several practical problems making the research more complex for several institutes to carry it out in parallel.

Chapter 3 will specifically focus on the release and complexation processes of PAHs in the intestinal lumen, which is important for a proper assessment of the bioaccessible fraction of ingested soil-bound PAHs. This part of the research will deal with several parameters that are playing a keyrole in the bioaccessibility process and will describe a mathematical model which can be specifically used for the PAH contaminated soil which was investigated. The subsequent chapters will deal with the microbial processes that may be related to PAH toxicity in the intestinal lumen. Based on preliminary bioassay data, there were good indications that colon microbiota were capable of transforming PAHs into more bioactive compounds, more in particular with estrogenic activity.

Chapter 4 describes the development and optimization of an analytical method using liquid chromatography coupled to mass spectrometry (LC-MS) to screen for microbial PAH metabolites that may have estrogenic properties, more specifically hydroxy-PAHs.

Chapter 5 focuses on the estrogenic activity of the PAH metabolites. Using two bioassays, the aryl hydrocarbon receptor yeast assay and the estrogen receptor yeast assay, the affinity of PAH parent compounds and hydroxy-PAH metabolites for human receptors will be measured. This will be brought in direct relationship with the PAH

bioactivation potency of intestinal microbiota, an aspect that has not been investigated thus far in HHRA.

Chapter 6 reports that supplementation of inulin, a much studied prebiotic compound, not only has prebiotic effects, but also a chemopreventive action. More in particular, it will be shown that the PAH bioactivation potency of the colon microbiota is largely inhibited by the indirect effects that inulin supplementation purports in the colon lumen.

Chapter 7 will give a general discussion of the different research chapters and deliver some take home messages that have come out of the research. Additionally, important links can be made with future scientific research which will study the contribution of luminal processes in the intestine, including microbial metabolism, to the total risk of ingested contaminants, not only from soil, but also from nutrition. An important link with the relationship between the intestinal microbiota and the intestinal epithelial wall can be brought forward and open perspectives for new research initiatives.

All experiments in this PhD research were carried out with the aid of a SHIME reactor (Figure 1.15). Batch experiments with SHIME specific parameters were used to perform the large number of digestion experiments. A full-scale SHIME run was applied to monitor shifts in the microbial community in Chapter 6.

Figure 1.15 Laboratory setup of the Simulator of the Human Intestinal Microbial Ecosystem at LabMET.

CHAPTER 2

Comparison of five in vitro models of the human gastrointestinal tract to assess lead bioaccessibility

Abstract

This paper presents a multi-laboratory comparison study of *in vitro* models assessing bioaccessibility of soil-bound lead in the human gastrointestinal tract under simulated fasted and fed conditions. Bioaccessibility is an important prerequisite to oral bioavailability. Yet, the relation between these two parameters is not wel understood. Therefore, the bioaccessibility results of this round-robin were compared with bioavailability data from a previous human *in vivo* study in which the same soil was investigated. In general, the bioaccessible lead fraction was significantly ($p<0.05$) different for each *in vitro* method and ranged for the fasted models from 2 % to 33 % and for the fed models from 7 % to 29 %. The *in vivo* data from literature were 26.2 % for fasted conditions, compared to 2.5 % for fed conditions. Three *in vitro* methods gave higher lead bioaccessibility values under fed conditions whereas two other methods showed the reverse trend, the latter in analogy with the *in vivo* study. The separation step for estimating bioaccessible lead (centrifugation speed, filtration, dialysis) was of major influence for the interpretation of the results. The use of ultrafiltration membranes mimics the *in vivo* data more closely, both under fasted as fed conditions. We conclude that the relationship between bioaccessibility and bioavailability depends on both the used *in vitro* model as the separation tool to assess bioaccessibility. It appears that the use of hollow-fibre membranes may decrease the correction factors needed to link *in vitro* to *in vivo* results.

***Redrafted after**: Van de Wiele TR, Oomen AG, Wragg J, Cave M, Minekus M, Hack A, Cornelis C, Rompelberg CJM, De Zwart LL, Klinck B, Van Wijnen J, Verstraete W, Sips AJAM. Comparison of five in vitro digestion models with in vivo data: lead bioaccessibility in the human gastrointestinal tract. Submitted.*

Introduction

Lead is a wide spread heavy metal in the environment and toxic to the human body by both chronic or acute exposure. The nervous system is the most sensitive target of lead exposure (Rosen, 1995), but basically, lead can affect every organ system, leading to anemia, renal problems and hypertension (ATSDR 1999). Lead can also decrease sperm count and increase abnormal sperm densities (Alexander et al., 1996; Telisman et al., 2000). Exposure to lead primarily occurs through inhalation and ingestion of contaminated matrices. Unfortunately, the soil and dust of urban areas with their associated high traffic and industrial density have higher lead concentrations than remote areas (ATSDR, 1999).

Oral exposure not only to lead, but other environmental contaminants via soil ingestion is an important public health issue. Site-specific risk assessment studies incorporate soil ingestion with a maximal daily intake of 50 and 150 mg soil d^{-1} for adults and children, respectively (EPA, 1997). Reported human soil intake rates generally range from 1 to 50 mg d^{-1} for adults and from 100 to 500 mg d^{-1} for children (Brunekreef et al., 1987; Vanwijnen et al., 1990; Calabrese et al., 1997a). However, *pica* afflicted children may show an unusual hand-to-mouth behavior and can ingest several grams up to 20 g soil d^{-1} (Calabrese et al., 1997a). Estimating the health risk associated with ingestion of contaminated soils requires an understanding of how contaminants are solubilized in the intestinal suspension and which fraction is considered for intestinal absorption.

The concepts of bioaccessibility and oral bioavailability are fundamentally important for investigating oral exposure to environmental contaminants. Bioaccessibility refers to the fraction of a contaminant that is released from soil into solution by digestive juices. It gives the maximum amount of contaminant that is available for intestinal absorption. In general, only a fraction of these bioaccessible contaminants can be absorbed by the intestinal epithelium and subsequently transported to the liver for biotransformation processes. The fraction of parent compound that reaches the systemic circulation is referred to as the bioavailable fraction (Figure 2.1). The two principal factors limiting the bioavailable fraction of a contaminant, are dissolution in the gastrointestinal tract and the actual intestinal absorption. In current risk assessment practice for environmental contaminants, oral doses from various exposure routes are compared with toxicological reference values based on intakes from water or food matrices. Soil bound contaminants, however, have different desorption and complexation processes

in the gastrointestinal tract than those found in food or water. These processes are suspected to influence their bioaccessibility (Oomen et al., 2002).

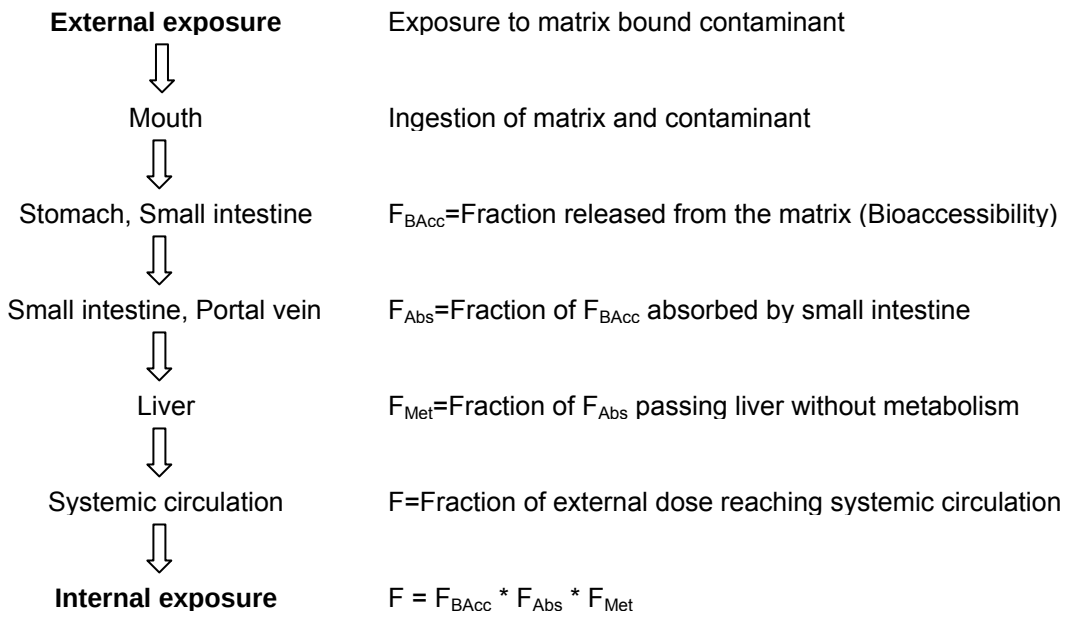

Figure 2.1 Overview of how ingested matrix-bound contaminants may become bioavailable in the gastrointestinal tract

Human bioavailability data from actual human feeding tests are scarce (Maddaloni et al., 1998) and *in vivo* experiments in general are costly, time consuming and related to ethical constraints. An alternative is the application of *in vitro* models that simulate the human gastrointestinal tract. These screening methods are fast, reproducible and reliable and can be used to measure the bioaccessible contaminant fraction, as bioaccessibility is an important parameter prior to bioavailability. Several *in vitro* methods of the human gut have been developed (Molly et al., 1993; Minekus et al., 1995; Ruby et al., 1996; Hack and Selenka, 1996; Jin et al., 1999; Oomen et al., 2002) and validated. Some of these methods have already been applied to measure bioaccessibility of both heavy metals and organic compounds (Hack and Selenka, 1996; Ruby et al., 1996; Oomen et al., 2000; Ruby et al., 2002). In a previous paper, we compared five *in vitro* digestion models that were applied on standard reference soils contaminated with arsenic, cadmium and lead (Oomen et al., 2002). However, no clear association exists between the bioaccessible fraction assessed in these tests and human bioavailability data. The purpose of this research was to compare *in vitro* bioaccessibility estimates with *in vivo* data. To accomplish this we used five different *in vitro* digestion models to estimate lead bioaccessibility from the Bunker Hill soil and compared these results to those of Maddaloni et al. (1998) who measured oral lead

bioavailability in adults for the same Bunker Hill soil. Similar to the *in vivo* study, we investigated fasted and fed conditions in the gastrointestinal tract. Our intention was not only to evaluate the different *in vitro* methods but also to highlight areas in which the models should converge to best mimic *in vivo* results. This comparison study could improve our understanding of how to relate bioaccessibility to oral bioavailability of ingested soil contaminants.

Materials and Methods

Soil. The standard reference Bunker Hill soil was kindly provided by Mark Maddaloni. It contains mostly clay and 3% organic matter and has a pH-H_2O of 5.5. The bioavailability of Pb in Bunker Hill soil to humans has been determined in an *in vivo* study by Maddaloni et al. (Maddaloni et al., 1998). Bunker Hill soil in the present study has not been subjected to γ irradiation for sterilization.

Experimental design. The bioaccessibility of Pb in the Bunker Hill soil was assessed with five *in vitro* digestion models. Four of the models are so called 'static models': the modified PBET method (Physiologically Based Extraction Test) operated by the BGS (United Kingdom); the German E DIN 19738, applied by the Ruhr-Universität Bochum (Germany); the RIVM (the Netherlands) in-vitro digestion model and the SHIME procedure used by LabMET (Ghent University, Belgium). The only dynamic gastro-intestinal model used is the TIM method (TNO, the Netherlands). The models are described in more detail below. Maddaloni et al. (1998) investigated two scenarios in their bioavailability study of a lead contaminated soil: fasted conditions in which soil was ingested with water upon overnight fasting and fed conditions where soil was ingested with a standard breakfast meal. In this study, fasted and fed conditions were applied for every *in vitro* model too. However, the composition of the nutrition was in most cases not the same as that used in the *in vivo* study, since the intrinsic intentions and concepts of the methods (for example simulation of the child's gut) were left unchanged.

Pb analysis. Pb analysis of the digestive juices, the pellets and the Bunker Hill soil was performed by the Flemish Institute for Technological Research (VITO, Belgium). This way, possible differences in bioaccessibility values that would originate from the different analytical methodology to measure the lead content in the samples were eliminated. The Bunker Hill soil was analyzed in triplicate for lead content using a

closed microwave oven destruction with HCl/HNO_3 (3+1) and ICP-AES quantification. The pellets from the *in vitro* bioaccessibility tests were dried until constant weight; lead determination was performed the same way as for the soil analyses. Liquid samples were digested by semi-open microwave oven destruction with HCl/HNO_3 (3+1) and ICP-MS or ICP-AES determination depending on the lead concentration in solution. Blank digestion solutions were analyzed as a control.

Description of *in vitro* digestion models.

PBET (Physiologically Based Extraction Test), BGS, United Kingdom. (Ruby et al., 1996). 1.0 g of soil was weighed into wide mouthed HDPE (high density polyethylene) bottles. 100 mL of simulated gastric solution (1.25 g pepsin, 0.50 g sodium malate, 0.50 g sodium citrate, 420 µl lactate and 500 µl acetate per liter de-ionised water, adjusted to pH 2.5 with concentrated hydrochloric acid) was added to each bottle. The bottles were placed in a water rotator set at 37°C. For the fed and fasted experiments, 1 g of Bunker Hill soil was extracted in triplicate for each method. After one hour at 37°C, a 5.0 mL aliquot was removed and filtered through a 0.45 µm cellulose filter disk for analysis. This extraction sample is known as the stomach phase. Five mL of the original gastric solution was then back-flushed through the filter into the HDPE bottle to retain the original L/S ratio (liquid (mL) to soil (g) ratio). The conditions in the vessel were then altered from stomach to small intestinal conditions by titration to pH 7.0 with saturated sodium bicarbonate and the addition of 175 mg bile salts and 50 mg pancreatin. The samples were then incubated in the water bath for four hours. These samples represented the small intestine. The experiments to simulate fed state included the addition of 1.0 g of baby whole milk powder (Cow & Gate, UK) to the digestive suspension. The composition of this whole milk powder was (47% carbohydrates from which 3% fibre, 45% lipids and 8% proteins).

Method E DIN 19738, Ruhr-Universität Bochum, Germany.

The German method E DIN 19738 has its origin in the *in vitro* digestion models of Rotard et al. (Rotard et al., 1995) and of Hack and Selenka (Hack and Selenka, 1996). It is a static gastrointestinal model that uses synthetic digestive juices. Since it is assumed that saliva has a negligible effect on the level of mobilization of contaminants from soil, only synthetic gastric juice and synthetic intestinal juice was used in the present round robin. Whole milk powder (50 g L^{-1}) was added to the gastric juice to simulate the influence of food on the mobilization of contaminants. The composition was 40% carbohydrates, 26% lipids and 26% proteins. Two g of contaminated dry soil were suspended in 100 mL gastric juice for two hours at pH 2.0. This was followed by

the addition of 100 mL of intestinal juice, the pH was set to 7.5, and digestion proceeded for six hours. The temperature was controlled by means of a water bath (37 °C). Mixing occurred with an agitator at 200 rpm. The digestion mixture was centrifuged for ten minutes at 7000 g, after which the supernatant was decanted. The residual pellet was stirred in 30 mL of distilled water for 0.2 h, centrifuged, and the supernatant decanted. The decanted intestinal solutions were combined for analysis.

In vitro digestion model, National Institute of Public Health and the Environment (RIVM), The Netherlands.

The fasting model and the composition of its digestive juices have been described in detail by Oomen et al. (Oomen et al., 2003). Briefly, the digestion was started by addition of 9.0 mL saliva of pH 6.5 to 0.6 g dry matter soil. This mixture was rotated end-over-end at 55 rpm at 37 ºC. Then, 13.5 mL gastric juice of pH 1.07 was added, and rotated at 37 ºC. After two hours, 27 mL duodenal juice (pH 7.8) and 9 mL bile juice (pH 8.0) were added. This mixture was rotated at 37 ºC for 2 hours and subsequently centrifuged at 3000g for 5 minutes. The supernatant (total volume 58.5 mL) represented the chyme. For the fed model, 6 mL of simulated saliva (pH 6.8), 4.5 g of infant formula (macaroni based), and 12 mL of stimulated gastric juice (pH 1.30) were added to 0.4 g dry matter soil. The mixture was rotated end-over-end at about 55 rpm at 37 ºC for 2 h. Subsequently, 12 mL of stimulated duodenal juice (pH 8.1), 6 mL of stimulated bile juice (pH 8.2) and 2 mL $NaHCO_3$ (85 g L^{-1}) were added. The latter was to adjust the pH of chyme to 6.5-7.0. After 2 h incubation, the chyme was separated from the pellet by centrifugation at 3000 g. For both models, the pH was determined at the end of the stomach and of the intestinal phase. The composition of the non-stimulated digestive juices is based on human physiology and is described in more detail (Oomen et al., 2003). Stimulated saliva contains more bicarbonate, α-amylase and less mucine than non-stimulated saliva. Stimulated gastric juice contains more pepsine, whereas stimulated duodenal and bile juice contains more pancreatine and lipase and five times more bile than non-stimulated duodenal juice.

SHIME (Simulator of Human Intestinal Microbial Ecology), LabMET (Ghent University, Belgium).

The SHIME procedure as employed in the present set-up represented a static gastrointestinal system simulating the gut of young children. The different digestive juices were added to the same reactor. The system can be extended to more reactors in which case a dynamic system with larger volumes and pH gradients can be obtained. The gastrointestinal model can also be extended with a compartment

representing the colon by introduction of colon microbiota into the system. This possibility was not applied in this study.

Five grams of soil were introduced into 50 mL of SHIME nutritional medium, which had a starting pH of 5.2 and this was supplemented with pepsin. The gastric pH was brought to 2.0 for fasted conditions and to 4.0 for fed conditions. SHIME nutritional medium contains per liter sterile distilled water 15 g Nutrilon plus, 16 g pectin, 8 g mucin, 5 g starch, 1 g cellobiose, 1 g glucose and 2 g proteose peptone. Nutrilon is nutrition for children between 4 and 18 months, and is obtained from Nutricia (Bornem, Belgium). Main constituents are lactose (56 %), fat (12 %), and casein (10 %). After 3 hours of incubation at 37 °C, 25 mL of a solution of pancreatic enzymes and bile salts was supplemented in order to obtain small intestinal fluid. This solution consists of 12 g $NaHCO_3$, 4 g bovine bile and 0.9 g pancreatine per liter of distilled water. This small intestinal suspension had pH 6.5, and was stirred at 150 rpm at 37 °C for 5 hours. Subsequently, the samples were centrifuged for 10 minutes at 7000 g, after which pellets and supernatants were analyzed. When fed conditions were simulated, 200 mL of gastric juice were supplemented to 5 g of soil. For the small intestinal digestion, 100 mL of the enzymatic and bile solution were dosed to the gastric suspension.

TIM (TNO gastro-Intestinal Model), TNO, The Netherlands.

The TNO model is a dynamic model that simulates the pH profile as well as continuous addition of enzymes, bile, and other components during gradual transit of soil through the different compartments of the gastro-intestinal tract (Minekus et al., 1995). The amount of soil and the meal was based on the study presented by Maddaloni et al. (Maddaloni et al., 1998). One hundred milligrams of soil were introduced in the model with 240 mL of water or the standard breakfast from the *in vivo* study to study the fasted and fed situation, respectively. Briefly, the main constituents of the standard breakfast were 25 g of wheat cereal, 130 g of whole milk, 1 large hard-boiled egg (approximately 48 g), 50 g of firm whole wheat bread, 6 g of butter, 5 g of jam/preservatives, and 6 g of white sugar. The nutritional composition was 50% carbohydrates, from which 15% fibre, 20% lipids and 30% proteins. The halftime of gastric emptying was 30 min for the fasted and 80 min for the fed situation. The initial gastric pH during fed situation was 5 and gradually decreased to 3.5, 2.5 and 2 after 30, 60 and 90 minutes, respectively. During the fasted situation, the gastric pH started at pH 4.5 whereafter it decreased to 3.2, 2.8 and 1.8 in 10, 20 and 40 minutes, respectively. Subsequently, the soil was gradually transferred to the intestinal compartments, representing the duodenum (pH 6.5), the jejunum (pH 6.8) and the ileum (pH 7.2). The gastric and duodenal secretion was set to 0.5 and 1 mL min^{-1},

respectively. The total digestion time is 360 minutes. The chyme is mixed and transported by peristaltic movements. Dialysis membranes (Hospal, MWcutoff 5000-10000) are used to remove bioaccessible contaminants, digestive metabolites and water from the chyme.

Round robin.

Experimental design. The bioaccessibility of Pb in the Bunker Hill soil was assessed with the *in vitro* digestion models described above. There were 4 static models: PBET, DIN, RIVM method and SHIME and 1 dynamic model: TIM. The soil was distributed by the RIVM institute, which ensured that the input material in the different digestion models was the same. Each of the institutes applied their *in vitro* model on the soil, both with fasted conditions as with fed conditions. The PBET and RIVM models measured lead bioaccessibility in both the stomach compartment alone as the stomach/intestine compartments combined. The RIVM method also performed digestion experiments at two different L/S ratios, 100 and 1000. Bioaccessibility was calculated as:

$$Bioaccessibility(\%) = \frac{lead\ mobilized\ \ from\ soil\ during\ digestion\ (\mu g)}{lead\ \ present\ in\ soil\ before\ digestion\ (\mu g)} * 100$$

Contaminant concentrations in the chyme and pellet were determined. This allowed a mass balance for each of the methods to be calculated.

Statistical information.

Standard practice for comparing different tests is to use analysis of variance (ANOVA). However, since there are relatively few data points for the methods used in this study, there are different numbers of replicate measurements for each test and there is no guarantee of the data being normally distributed, a simpler approach to a statistical comparison of these tests was to use a permutation test (Resampling Test software, 2001). In this instance the null hypothesis is that there is no significant difference in the bioaccessibility of lead as measured by the different methods. In order to test this, the individual mean values for each method are calculated along with the overall mean of all the tests being considered. The absolute differences between each test mean value and the overall mean are then calculated and summed. This provides the benchmark statistic. The data for all the tests are then randomly shuffled (retaining the same number of data points for each method) and the test statistic is recalculated. This process of random shuffling and calculation of the test statistic is repeated 10000 times recording the test statistic each time. The number of times the test statistic

exceeds the benchmark statistic is recorded. If there is no significant difference between the methods then test statistic is likely to exceed benchmark statistic in a large proportion of the reshuffling trials. If, however, there is a significant difference between the methods then it is unlikely that a randomly shuffled trial will exceed the benchmark statistic. The probability cut-off chosen in this study is that of 5 % (i.e. p=0.05). Therefore if 5 % or more of the trials are greater than the benchmark statistic then the null hypothesis is confirmed and there is no significant difference between the methods. If there are less than 5 % of the reshuffled trials greater than the test statistic then the results of the different methods are significantly different. The advantage of using this approach is that it is simple to carry out (calculations were carried out in Excel using Resampling Stats software, 2001) and no assumptions about the distributions of the data need to be made.

RESULTS

Bioaccessibility data from the different *in vitro* methods and bioavailability data, obtained from Maddaloni et al. (1998), were largely dependent on the absence or presence of food components. Figure 2.2 shows a comparison of the differences between the average bioaccessibility/bioavailability for the fasted and fed conditions. The resampling test showed that, apart from the RIVM stomach extraction for both 1/100 and 1/1000 liquid to solid ratios, these differences are significant. In analogy with the human bioavailability data, the TIM model showed much lower bioaccessibility results when fed conditions were simulated. The same trend was observed for PBET stomach, RIVM intestine 1/100 and RIVM intestine 1/1000, but to a lesser extent. Higher bioaccessibility data for fed conditions were obtained with the PBET intestine method, the SHIME and DIN method. The presence of food components in the digest suspensions thus plays a major role for the outcome of the bioaccessibility results.

Our quality assurance and quality control steps found that the lead concentration in the Bunker Hill soil was 3060 ± 55 mg Pb kg^{-1} soil similar to the value of 2924 ± 36 mg Pb kg^{-1} soil (dry weight) from the *in vivo* study (Maddaloni et al., 1998). In addition, lead recoveries from the fasted digestion types ranged from at least 92 % up to 98 %. The fed digestion types delivered lower recovery data ranging from 73 % to 93 % (Table 2.1). It should be noted that the lead analysis was performed by one analytical laboratory with all participants sending their samples to this institute for analysis.

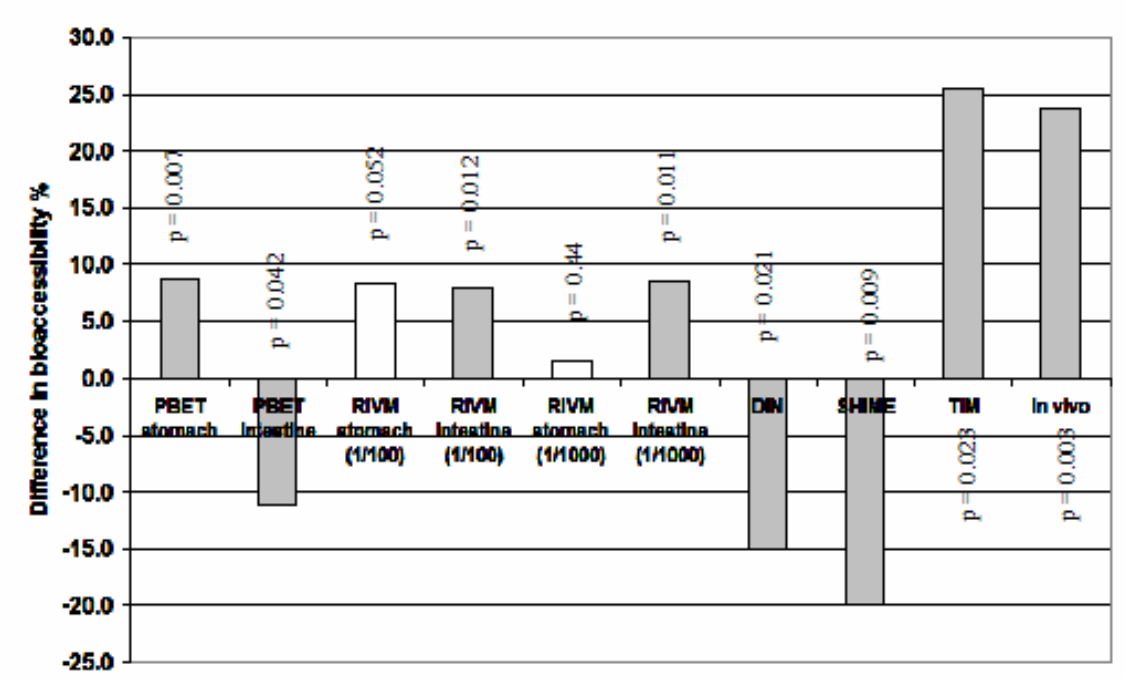

Figure 2.2 Difference in bioaccessibility (bioavailability for the *in vivo* study (Maddaloni et al., 1998)) between fasted and fed condtions (p values < 0.05 indicate a significant difference between fed and fasted). Grey bars are significant, white bars are not significant. A positive value means that bioaccessibility was higher under fasting conditions than under fed conditions.

Table 2.1 Lead recovery percentage, liquid to soil ratio and bioaccessibility results (± standard deviation) for the different *in vitro* gastrointestinal digestions of Bunker Hill Soil (2924 ± 36 mg Pb kg^{-1} soil DW)

	n	% recovery	L/S	% Bioaccess.
PBET				
Fasted	3	98	100	13.0 ± 0.8
Fed	2	93	100	21.8 ± 0.4
DIN				
Fasted	3	96	100	13.6 ± 0.6
Fed	3	88	100	28.6 ± 1.6
RIVM				
Fasted	3	90 ± 2	98	31.8 ± 2.5
Fed	3	78 ± 8	101	23.9 ± 2.4
SHIME				
Fasted	2	92	15	2.0 ± 0.1
Fed	3	83	62	24.1 ± 0.1
TIM				
Fasted	2	89	51	32.5 ± 4.5
Fed	2	73	51	7.0 ± 1.5

Fasted conditions. The *in vitro* methods differed significantly from each other and the *in vivo* data (p=0.003) (Figure 2.3). The SHIME method showed the lowest bioaccessibility value (2.0 %), followed by PBET intestine (13.0 %) and DIN (13.6 %), whereas the RIVM intestine method (31.8 %) and TIM (32.5 %) displayed higher bioaccessibility values (Table 2.1), quite comparable with the oral bioavailabitiliy value of 26.2 %. Lead bioaccessibility values from the PBET (25.0 %) and RIVM (70.9 %) stomach digests were higher than the respective small intestine and stomach digests combined. RIVM also performed digestions at two different liquid to soil (L/S) ratios, being approximately 1000:1 and 100:1 (Table 2.2). L/S ratios of 1000 caused higher bioaccessibilities, 85.9 % and 47.4 % for stomach and small intestine respectively, compared to digests at a L/S ratio of 100, 70.9 % and 31.8 % for stomach and small intestine, respectively. It should be emphasized that bioaccessibility was assessed differently in each model and that this bioaccessibility value is not physiologically directly linked to the blood values (=bioavailability) obtained in the *in vivo* study (Maddaloni et al., 1998).

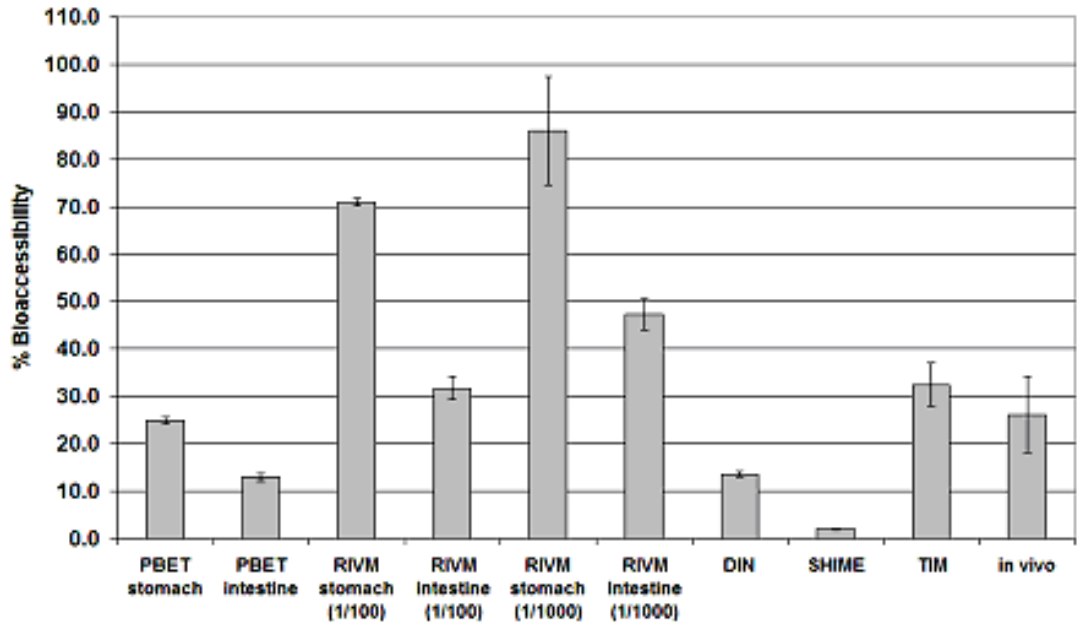

Figure 2.3 Comparison between methods of the bioaccessibility data upon simulated fasted conditions and the bioavailability data from the *in vivo* study (Maddaloni et al., 1998). Bars show the range of results that were obtained with different replicates within one method.

Table 2.2 Bioaccessibility results (± standard deviation) from the PBET and RIVM models upon stomach digestion. The RIVM method also obtained bioaccessibility data with L/S ratios of 1000

	L/S	% Bioaccess. Stomach	% Bioaccess. Intestine
RIVM method			
Fasted	975	85.9 ± 11.4	47.4 ± 3.2
	98	70.9 ± 0.9	31.8 ± 2.5
Fed	1013	84.4 ± 4.5	38.8 ± 1.9
	101	62.5 ± 7.4	23.9 ± 2.4
PBET			
Fasted	100	25.0 ± 0.7	13.0 ± 0.8
Fed	100	16.2 ± 0.2	21.8 ± 0.4

Fed conditions. Significant differences (p=0.0008) between all methods, including the *in vivo* study by Maddaloni et al. (1998), were obtained. The bioaccessibility values from all small intestinal digestions, except for TIM, were higher than the *in vivo* bioavailability results (Figure 2.4). The DIN method had the highest bioaccessibility value (28.6 %), whereas the digestion models SHIME, RIVM and PBET produced slightly lower values of 24.1%, 23.9% and 21.8 %, respectively. These bioaccessibility results were significantly different from the bioavailability data. The TIM method on the contrary returned the lowest fed bioaccessibility value, 7.0 %, quite comparable with the *in vivo* bioavailability of 2.5 %. The stomach bioaccessibility results for the RIVM method at L/S ratios of 100 (62.5 %) and 1000 (84.4 %) were higher than the small intestine bioaccessibilities (23.9 % and 38.8% respectively). The PBET method simulating fed conditions returned a lower bioaccessibility value in the stomach (16.2 %) than in the small intestinal digest (21.8 %), this in contrast to the fasted conditions of the PBET method in which the opposite was observed.

Static versus dynamic models. The bioaccessibility results obtained with the dynamic TIM model were not significantly different from the *in vivo* bioavailability data, both under fasted and fed conditions. Concerning the variability of the bioaccessibility results, we observed low RSD values for all *in vitro* methods compared to high RSD values of 31 and 66 % for the *in vivo* data obtained from fasted and fed conditions, respectively. Generally, the static models PBET, DIN, SHIME and RIVM returned RSD values below 10 % whereas the dynamic TIM model showed slightly higher RSD values of 14 and 21 % for fasted and fed conditions, respectively. These variabilities show that the bioaccessibility measurements with all methods are quite reproducible.

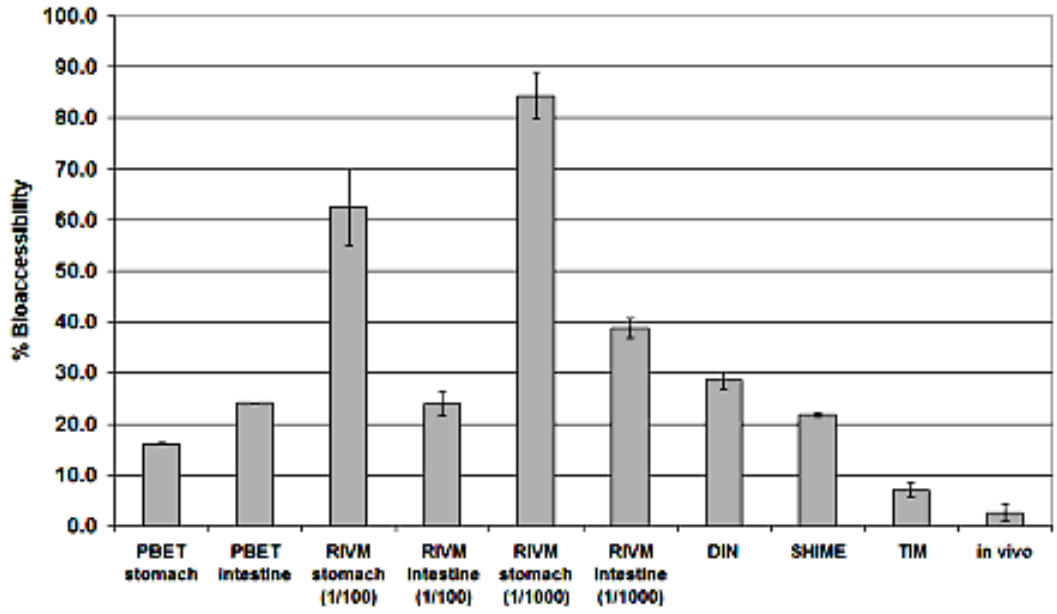

Figure 2.4 Comparison between methods of the bioaccessibility data upon simulated fed conditions and the bioavailability data from the *in vivo* study (Maddaloni et al., 1998). Bars show the range of results that were obtained with different replicates within one method.

Discussion

***In vitro – in vivo* comparison**. Since the bioaccessibility process is an important precursor to bioavailability, *in vitro* methods should always return bioaccessibility values that are higher than the bioavailability values from *in vivo* experiments on the same soil. The *in vitro* methods appeared to be a conservative estimator of lead bioavailability under fed conditions, because higher or similar bioaccessiblity values were obtained in comparison with bioavailability values (Figure 2.4). Under fasting conditions, methods such as the PBET intestine, DIN and SHIME methods under-estimated bioavailability whereas the PBET stomach, RIVM and TIM models gave higher bioaccessibility than *in vivo* bioavailability results (Figure 2.3). Upon simulated fed conditions, only the RIVM and TIM methods showed a decrease in bioaccessibility compared to fasted conditions, similar to that seen with the *in vivo* bioavailability data (Figure 2.2). To partly explain some of the discrepancies, we should keep in mind that the PBET, DIN, RIVM and SHIME methods specifically simulate the gastrointestinal tract of small children, whereas Maddaloni et al. (1998) arrived to bioavailability data from adults. The differences between the *in vivo* and *in vitro* results also arise because the *in vitro* approaches measure bioacccessibility and the *in vivo*, bioavailability of lead

from the ingested soil. Clearly, those two parameters are different and we should have a clear view on how bioaccessibility is brought into relationship with bioavailability.
As introduced earlier, the bioaccessible contaminant fraction is this fraction which is released from the soil, solubilized in the gastrointestinal tract and is available for intestinal absorption (Figure 2.1) (Ruby et al., 1996; Oomen et al., 2000; Hamel et al., 1998). Bioaccessible contaminants are partly absorbed through the small intestinal wall and subsequently reach the blood stream after which they can be biotransformed in the liver. For lead, hepatic biotransformation is however not an issue. The lead fraction that actually reaches the blood stream is considered as bioavailable. Bioaccessibility is thus an important prerequisite to bioavailability, hence a higher bioaccessible contaminant fraction should be obtained *in vitro* than the bioavailable fraction. In general, an *in vitro* approach will only be reliable if liberation from soil in the gastrointestinal tract is the limiting step in the oral bioavailability process (Ruby et al., 2002). For the simulated fed conditions, all *in vitro* methods are applicable for risk assessment since they return higher bioaccessibility values than *in vivo* bioavailability data. The relationship with the bioavailable fraction can be made, provided a correction factor should link bioaccessibility with bioavailability. We should keep in mind that this correction factor will be different for every single method, since different tools are used to measure bioaccessibility.

Presence of food components. The presence of food components was of significant influence on the outcome of the bioaccessibility results (Figure 2.2). The DIN, PBET and SHIME gastrointestinal models gave higher bioaccessibility values during fed conditions than during fasted conditions, whereas the RIVM and TIM-model showed the reverse trend. Maddaloni et al. (1998) observed that lead bioavailability decreased upon soil ingestion together with a standardized breakfast meal. A decrease in intestinal lead absorption was already described when lead was complexed to rhamnogalacturonan-II dimers, a pectide polysaccharide of the cell wall of fruits and vegetables (Tahiri et al., 2000). The presence of nutrition in the gastrointestinal juice means that more dissolved organic matter is present, providing more complexation niches for lead in solution. However, this also means that lead is in a complexed state with solubilized organic material, rather than in a free absorbable state. This will be reflected in a lower fraction of released lead that can actually be considered for small intestinal absorption. Obviously, intestinal absorption is not considered by any of the *in vitro* methods, but preceeding steps to intestinal absorption are considered when the bioaccessible lead fraction is determined. The PBET, DIN and SHIME methods use whole milk powder or milk based nutrition that are rich in fat

content. Fat globules can take up desorbed lead from the soil matrix and thus increase the lead fraction in the intestinal suspension. However, the 0.45 µm filtration or 7000×g centrifugation may not be stringent enough to separate these lead-dissolved organic matter complex. The nutrition used in the RIVM and TIM models was also fat rich – TIM even used the same breakfast meal as the *in vivo* study – but was also higher in carbohydrate content, especially the breakfast meal that was rich in food fibres due to the presence of whole grain bread and cereals. Fibre/lead complexes are larger than lead complexes with whole milk fat globules and can therefore be more easily separated. This may be reflected in the RIVM data where lower lead bioaccessibility with fed conditions was obtained after a 3000×g centrifugation. Yet, RIVM arrived to a bioaccessibility as high as that obtained with the other methods (Table 2.1). The TIM method on the contrary, gave quite similar bioaccessibility data as the bioavailability data. Models that use ultrafiltration membranes as a separation step will remove more lead-dissolved organic matter complexes from bioaccessibility samples and will probably approach bioavailability more closely. Therefore, our data suggest that ultrafiltration membranes may be a technology that could be implemented for all digestion models to better mimic the effects of food on contaminant bioavailability.

Other parameters of influence. The static *in vitro* methods of the GI tract simulated conditions of the infant's gut, whereas the data from the dynamic TIM model and the *in vivo* bioavailability data from Maddaloni et al. (1998) were derived from adults. An important difference in digestion parameters can be noted. Firstly, the stomach pH of infants under fasted conditions (pH 3) is not as low as that of adults (1.5). This has important consequences on the release of metals such as lead from a soil matrix. Secondly, the amount of secreted digestive juices for an infant's gut is lower than that of an adult, which will lead to lower L/S (liquid to soil) ratios. This will influence the equilibrium between complexed and released contaminants in the chyme. Given the large discrepancies between the different models, it would be more useful for future experiments to mimick the gut of adults, rather than using the current digestion parameters which were developed to simulate the infant's gut. This would make an adequate comparison with *in vivo* data more possible.

The L/S ratio had an influence on the bioaccessible lead fraction, as shown by the results from RIVM (Table 2.2). Higher bioaccessibility values were obtained from soil digests using L/S ratios of 1000, compared to soil digests where L/S ratios of 100 was applied. These data do not correspond to the statement that bioaccessibility of metals mobilized from soils in *in vitro* synthetic gastric juice are only slightly influenced by changes in gastric fluid L/S ratios for the range 100 to 5000 (mL g^{-1}) (Hamel et al.,

1998). However, this influence may also be site-specific and thus dependent on the soil characteristics. Digests with a very low L/S ratio of 15 were investigated using LabMET's SHIME method, which was applied as a model for children assuming a hypothetical ingestion of 1 g soil kg^{-1} BW. The low bioaccessibility compared to the *in vivo* bioavailability may be attributed to this parameter, since most other parameters such as method of separation, stomach pH and time of digestion were quite comparable to for instance the DIN method, which arrived at higher bioaccessibility values. A second parameter of influence is the pH of the gastric juice which has an important effect on lead desorption or complexation. Data from the PBET and RIVM method showed that higher lead bioaccessibilities were obtained in the stomach compared to the intestine. The respective pH of 1.5 and 1.1 support solubilization of lead ions into the aqueous solution. The SHIME method applied the highest pH (4.0) of all models under fed conditions. Quite remarkably, a much higher bioaccessibility was observed. The much higher L/S ratio of 60 of the SHIME method compared to that of the fasted model, L/S 15, may explain this shift in equilibrium towards the bioaccessible fraction, together with the presence of food components in the fed model. The residence time of the gastric and intestinal digestions differ amongst the different *in vitro* methods. Too few data were however obtained in which the effect of digestion time could be investigated in this study. The underestimation of the bioavailable fraction by the PBET method upon fasted conditions may be due to the short gastric digestion time of 1 hour in comparison with longer gastric digestion times of 2 to 3 hours with the other models.

Differences in bioaccessibility between models may also be explained by the different purposes for which the models were initially developed. Firstly, the PBET, DIN, RIVM and SHIME model are designed to simulate the gastrointestinal tract of small children, because children are regarded to be at higher risk of ingestion of contaminated soils, due to their hand-to-mouth behavior, than adults are. Secondly, most *in vitro* models are static, which means that the gastrointestinal transit of soil particles is simulated by sequential exposure to synthetic saliva, gastric and intestinal juices. The TIM method on the contrary, is a dynamic model. Dynamic models can simulate more aspects of the human physiology, for instance the pH profile as well as continuous addition of enzymes, bile, and other components during gradual transit of soil through the different compartments of the gastrointestinal tract. The SHIME is often applied in this manner but not in this study. These dynamic models are fairly sophisticated whereas static models are easy to use and allow simultaneous determination of a large number of samples. The TIM method is a more advanced *in vitro* model, simulating gradual transfer of matrix and contaminant to subsequent

gastrointestinal compartments, whereas the other models have applied the gastrointestinal digestion in a more general way. This model complexity is reflected in a slightly higher variability of the TIM results compared to that seen with the other *in vitro* methods. Although all *in vitro* methods from this study gave bioaccessibility values that were quite reproducible, the advantage of the static *in vitro* models is that they may be used as high through-put screening methods with which site-specific risk analysis can generate many results in a limited time frame.

Relationship between bioaccessibility and bioavailability. There are 2 principal limiting factors when the bioavailable fraction of an ingested contaminant is to be estimated, namely dissolution in the gastrointestinal tract and intestinal absorption. *In vitro* methods of the human gut do not simulate intestinal absorption and are therefore only reliable as risk assessment tool when contaminant release from the soil matrix is the rate limiting step in the oral bioavailability process (Ruby et al., 2002). The tool for measuring the bioaccessible fraction is however dependent on the used separation method. The PBET method applied a 0.45 µm filtration in analogy to Holman et al. (Holman et al., 2002), whereas the RIVM method separated the bioaccessible lead fraction during a 10 minutes centrifugation step at 3000×*g*, as suggested by Ruby et al. (1996). Both the DIN and SHIME methods used a 7000×*g* centrifugation step, whereas the TIM model used hollow fibre membranes with a molecular cut-off of 5 to 10 kDa. These differences are of critical importance when bioaccessibility data are to be interpreted. Some of the desorbed lead may however be present in complexes this large that intestinal absorption will not occur. From that instance, the rate limiting step for a compound to become bioavailable is not the soil desorption, but the complexation processes within the intestinal lumen. For this, each *in vitro* model uses correction factors to relate lead bioaccessibility to bioavailability. It is important and crucial for risk assessment to recognize that this correction factor will be different depending on the separation method that is used to measure bioaccessibility. Hence, interpretation of bioaccessibility data for risk assessment should not only consider what type of *in vitro* method is used to account for the different parameters and characteristics of the model, but should also make corrections, depending on which separation step is used. This separation step is directly related to investigating lead speciation in gastrointestinal fluids (Oomen et al., 2003). This will provide a more detailed understanding of complexation processes in the intestine and of the lead fraction that may be available for transport across the intestinal epithelium.

The results from this study indicate that most of the five methods gave fairly similar bioaccessibility results. The methods over-estimated bioavailability under fed

conditions and gave mixed results under fasting conditions. The use of hollow-fibre membranes such as those used in the TIM probably mimics the results from an *in vivo* study more closely. We hypothesize that this may be the ability of hollow-fibre membranes to reject large lead-organic matter complexes that form in the gastrointestinal tract. In conclusion, our results suggest that *in vitro* models are an effective way to mimic the human digestive process and that the addition of ultrafiltration as separation step may increase the accurateness of bioaccessibility measurements.

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CHAPTER 3

Polycyclic aromatic hydrocarbon release from a soil matrix in the in vitro g*astrointestinal tract*

Abstract

Soil ingestion is an important exposure route by which immobile soil contaminants enter the human body. We assessed polycyclic aromatic hydrocarbon (PAH) release from a contaminated soil, containing 49 mg PAH kg^{-1}, using a SHIME (Simulator of the Human Intestinal Microbial Ecosystem) reactor comprising the stomach, duodenal, and colon compartments. Polycyclic aromatic hydrocarbon release was defined as that fraction remaining in the digest supernatant after centrifugation for 5 min at 1500×g. The PAH release in the stomach digest was only 0.44% of the total PAH present in soil, resulting in PAH concentrations of 23 µg PAH L^{-1} chyme. The lower PAH releases in duodenum (0.13%) and colon (0.30%) digests, compared with the stomach digest, were thought to be attributed to combined complexation and precipitation with bile salts, dissolved organic matter, or colon microbiota. We studied these complexation processes in an intestinal suspension more in depth by preparing mixtures of 9-anthracenepropionic acid, a *Bacillus subtilis* culture, and cholin as model compounds for PAHs, organic matter, and bile salts, respectively. Bile salts or organic matter in the aqueous phase initially enhance PAH desorption from soil. However, desorbed PAHs may form large aggregates with bile and organic matter, lowering the freely dissolved PAH fraction in the supernatant. Using the model compounds, mathematical equations were developed and validated to predict PAH complexation processes in the gastrointestinal tract. Contaminant release and subsequent complexation in the gut is an important prerequisite to intestinal absorption and thus bioavailability of that contaminant. The data from this research may help in understanding the processes to which PAHs are subjected in the gastrointestinal tract, before intestinal absorption.

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Introduction

Soil ingestion is an important human and animal exposure route for environmental contaminants with typical human soil intake between 1 and 500 mg d^{-1} (Brunekreef et al., 1987; Vanwijnen et al., 1990; Calabrese et al., 1997a, 1997b; Stanek et al., 1997). Site-specific risk assessment studies incorporate soil ingestion with a maximal daily intake of 50 and 150 mg soil d^{-1} for adults and children, respectively (USEPA, 1997). However, high soil ingestion rates might impose a larger health risk for *pica*-afflicted children, who, due to their unusual hand-to-mouth behavior, occasionally ingest several grams of soil with reported intakes of 5 to 60 g d^{-1} (Calabrese et al., 1999). Concerning the toxicology of contaminants as such, the intestinal absorption and hepatic biotransformation of bioavailable xenobiotics have been intensively studied. However, the desorption and complexation processes before intestinal uptake are not well understood. Yet, these phenomena in the gut lumen require further study as they largely influence the extent of oral bioavailability of soil-bound contaminants. The released fraction of contaminants that is available for absorption is defined as the bioaccessible fraction.

Polycyclic aromatic hydrocarbons (PAH) are common hydrophobic soil contaminants with natural and anthropogenic sources (Freeman and Cattell, 1990; Van Metre et al., 2000). Depending on their physicochemical characteristics, such as aromaticity, these compounds exhibit toxic, mutagenic, and carcinogenic effects or (anti)-estrogenic activities (Mastrangelo et al., 1996; Szeliga and Dipple, 1998; Hirose et al., 2001; Mersch-Sundermann et al., 2001). During intestinal transit, the presence of food components (Hack and Selenka, 1996) or bile (Oomen et al., 2000) in the gastrointestinal (GI) tract modulates the release of hydrophobic contaminants. Hack and Selenka (1996) ascribe the higher release of organic pollutants to the more hydrophobic character that food components give to the aqueous solution. In a similar manner, soluble soil organic matter forms microscale hydrophobic environments in the aqueous phase and acts as a mobile sorbent for hydrophobic compounds like polychlorinated biphenyls (PCBs) and PAHs (Chiou et al., 1986; Jota and Hassett, 1991; Kögel-Knabner et al., 2000). Applying different bile salt and protein concentrations, Oomen et al. (2000) calculated specific release ratios for PCB in an in vitro digestive solution and found that PCB mobilization from a surrogate soil—consisting of 10% peat, 20% kaolin clay, and 70% sand and prepared according to Organisation for Economic CoOperation and Development (OECD) Guideline 207—was largely influenced by the presence of bile salt micelles. They characterized PCB

aggregates with bile salt micelles or proteins as determined in a supernatant after 5 min at 3000×g, as bioaccessible.

Investigating desorption processes in the gut of oligochaetes, other invertebrates, fish, rodents, and other mammals is useful for relevant tests of oral bioavailability (Loonen et al., 1997; Koganti et al., 1998; Oste et al., 2001; Willett et al., 2001; Gomot-de Vaufleury and Pihan, 2002; Yan and Wang, 2002). However, contaminant release and complexation processes inside the GI tract are unknown mechanisms in these in vivo studies. Moreover, given the discrepancies between the physiology of humans and other organisms, as well as the cost and time constraints associated with such studies, a more efficient alternative is desirable (Ruby et al., 1999). In vitro digestion models that mimic the human GI tract can elucidate these phenomena. These models, based on the physiology of humans, are generally more simple, less time-consuming, and especially more reproducible than animal tests. Such tests simulating stomach, small intestinal digestions (Hack and Selenka, 1996; Ruby et al., 1996; Jin et al., 1999; Oomen et al., 2000), and intestinal transport through Caco-2 tissue cultures have been described before (Oomen et al., 2001) and could provide a rapid and inexpensive method for developing more accurate exposure estimates for use in human health risk assessments. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) effectively models the human intestinal tract (Molly et al., 1993, 1994) and is an automated and robust multistage reactor that is amenable to bioavailability estimates. (Figure 3.1).

Figure 3.1 Picture of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Vessels 1 to 5 respectively simulate conditions from the stomach, small intestine, colon ascendens, colon transversum and colon descendens.

This computer-controlled dynamic model consists of five compartments, with each compartment vessel simulating a different part of the GI tract from the stomach and small intestine to the proximal and distal part of the colon (http://www.avecom.be/shime_i.html). The SHIME reactor differentiates itself from other in vitro intestinal models, because it comprises the entire GI tract taking into account the enzymatic processes in the stomach and duodenum and the different characteristics of the microbiota along the colon reactors. Models such as these allow one to investigate the biochemical and physical processes contributing to PAH release inside the human GI tract.

In this research, we investigated intestinal desorption processes of PAHs in the case of high soil ingestion rates. We subjected a PAH-contaminated soil from a recreational area to batch experiments from the SHIME model and monitored the released PAH amount as observed in the stomach, the first part of the small intestine (duodenum), and the large intestine (colon). Additionally, a mathematical model was developed from a model compound to help us understand how much PAHs and which PAH complexes were formed during digestion. This model was evaluated against observed data from the different digestion steps of a contaminated soil. Our intent was to combine digestion and mathematical models to elucidate release and complexation mechanisms of soil-bound contaminants inside the child GI tract and thereby give an indication of what fraction of soil-bound contaminants will be bioaccessible.

Materials and Methods

Soil Sampling and Characterization

The top 0 to 6 cm of a soil was sampled from a recreation park in Zelzate, Belgium, which was contaminated with PAHs after 20 yr of atmospheric deposition from nearby industry. The soil samples were dried for 24 h at room temperature, homogenized, and stored until analysis at 4°C in the absence of light. Small rocks and plant material were removed by sieving (2 mm) before analysis. Soil organic matter content was 3.3% and determined by loss of mass on ignition in a muffle oven at 360°C for 2 h (Storer, 1984). The soil pH (H_2O) was measured according to Black et al. (1965) using a 1:10 soil to water suspension and was 6.5. The soil contained 94% sand, 4% silt, and 2% clay and was classified as moderate wet sandy soil (Geo-Vlaanderen, Flemish Land Company; 51°12’26” N, 3°48’20” E).

Digestions

Three different digestion processes of the human GI tract were simulated and compared with one another. The amount of soil subjected to the different digests was 20 g.

Stomach model. In a first model, simulating the stomach, the effects of pH and liquid to soil ratios (L/S, volume/mass) were studied, as well as the influence of food components on PAH release. A salt solution (200 mL) containing 0.1 M $KHCO_3$ and 0.1 M NaCl was applied to 20 g of soil. This solution at L/S 10 was acidified with 1.3 mL 5 M hydrochloric acid to a final measured pH of 1.5 after which porcine pepsin (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 10 mg pepsin L^{-1} soil suspension. The soil suspension was stirred at 150 rpm for 2 h at 37°C. Acidity effects were evaluated by using only water as a diluent in the stomach. This treatment is comparable with the German standard method DIN 38 414 S4 for leachability of soils with water. Influence of L/S using the normal salt, acid, and pepsin concentrations was assessed using L/S of 2, 10, and 40. The amount of soil tested, 20 g, stayed the same. Large enough digest bottles were taken to provide the same stirring efficiency in all digests as a difference in mixing intensity could have influenced the release of PAHs from the soil matrix. The L/S of 10 and 40 correspond to a daily soil ingestion of 20 and 5 g, respectively, during fasting conditions. The L/S of 2 was not derived from physiological data, but served as an additional data point to study the influence of L/S on PAH release at low L/S. The influence of food was assessed by adding liquid nutrition at a L/S of 40. The nutrition consists of a carbohydrate-based medium containing, per liter: 5 g Nutrilon and 3 g potato starch (Nutricia, Bornem, Belgium), 1 g arabinogalactan, 2 g pectin, 1 g xylan, 0.4 g glucose, 4 g mucin and 0.5 g cystein from Sigma-Aldrich, 3 g yeast extract, and 3 g pepton from BD (Franklin Lakes, NJ). Nutrilon is nutrition for small children, which consists of lactose (56%), fat (12%), and casein (10%).

Duodenum model. Although intestinal bacteria are present in the last part of the in vivo small intestine (ileum), the duodenum digestion model focuses on enzymatic activities, bile salts, and dissolved organic matter (DOM) complexation processes. Twenty grams of soil, which had already gone through a L/S 10 stomach digestion without any centrifugation step, were supplemented with 100 mL pancreatic juice containing 12.5 g L^{-1} $NaHCO_3$ (resulting in a measured pH of 6.3), 6 g L^{-1} Oxgall (BD), and 0.9 g L^{-1} porcine pancreatin powder (Sigma-Aldrich). The different pancreatic juice components were added separately or in combination to differentiate between PAH release due to the presence of bile salts, proteins, or complexes with DOM or

particulate organic matter. These small intestinal soil suspensions were stirred at 150 rpm for 5 h at 37°C. The resulting L/S was 15.

Colon model. Mobilization mechanisms influenced by gut microbiota were investigated in the colon digestion model. The small intestine digest suspension containing 20 g of soil with an L/S of 15 was supplemented with 100 mL of SHIME suspension which contains microbiota in a representative concentration and composition for the human colon (Molly et al., 1993). Total anaerobe and aerobe concentrations were 8.4 and 7.8 log colony forming units (CFU) mL^{-1} and included microbiota such as Lactobacilli, Bifidobacteria, Enterococci, Fungi, Staphylococci, and Clostridia. This colon digest was incubated at 37°C and stirred at 150 rpm for 18 h. The resulting L/S was 25.

Sample treatment and PAH analysis

During the different digestions, PAHs can be mobilized and subsequently complexed with several compounds such as bile salts, DOM, or particulate organic matter. We used a 1500×g centrifugation speed during 5 min to spin down aggregates larger than 5 µm. This was calculated with the Stokes equation using the centrifugation parameters. Upon centrifugation, supernatants and pellets were analyzed for PAH content. In the colon model, bacteria were present, also. To account for PAH adsorption to bacterial biomass, the colon samples were subsequently centrifuged at 3000×g for 5 min, which roughly spun down small complexes with a 1.5 µm diameter as calculated with the Stokes equation (Stokes, 1851). Sample treatment for and determination of PAHs were performed by the Environmental Research Center (Erembodegem, Belgium). Briefly, PAHs from pellets were extracted by a 1:1 acetone and hexane mixture using an accelerated solvent extractor (Model 200; Dionex, Sunnyvale, CA). The PAHs from supernatants were extracted with dichloromethane. Analysis of the PAH content in the extracts was performed according to a standardized method (USEPA Method 8270) by gas chromatography coupled with mass spectrometry (GC–MS). The MS detector used was a quadropole mass spectrometer (Trace-MS; Thermo Finnigan, San Jose, CA). The detection limit for the different PAH components was 0.2 µg L^{-1}. The quantification limit was 0.4 µg L^{-1}. The extraction efficiency of the sample preparation step before PAH analysis was between 80 and 110%, as determined with the reference soil CRM535. Organic matter content in the supernatants was assessed according to standardized method NBN 357.02 (Greenberg et al., 1992). Briefly, this method consists of determining the loss of mass in the samples after they were heated at 600°C during 4 h.

Study of PAH complexation mechanisms

The diversity of components in the intestinal suspension makes the study of mobilization and complexation processes in the GI tract difficult. We considered the presence of bile salts, DOM, and particulate organic matter as key factors in the release and complexation of PAHs during digestion and selected a single model compound for each of these factors. We prepared 320 samples in which the three model compounds were mixed in different concentrations. The first model compound, indicated with the letter A, was a fluorescent PAH derivative, 9-anthracene propionic acid (CAS no. 41034-83-7; Molecular Probes, Leiden, the Netherlands). This molecule could be used for epifluorescence analysis as it absorbs light at 366 nm and emits at 414 nm. Due to the presence of the propionate group, the pKa value of this compound was 4.6 as calculated by Solaris Version 4.67 software (Advanced Chemistry Development, 2001). At a pH of 6, the log organic carbon partitioning coefficient (KOC) value was estimated to be 2.4, which was found hydrophobic enough to serve as model compound for low molecular weight PAHs that adsorb to organic matter. The solubility was estimated to be 0.497 mg L^{-1} (EPI Suite; EPA, 2004), which is higher than 0.0434 mg L^{-1} for anthracene. The 9-anthracene propionic acid was used at concentrations of 0.01, 0.5, 10, and 100 mg L^{-1}. To study interaction with organic matter, a culture of *Bacillus subtilis* was prepared as previously described (Daughney and Fein, 1998) and supplemented in concentrations of 0, 0.06, 0.12, 0.31, 0.61, 1.22, 1.83, and 3.05 g organic C L^{-1}. This organism has been used as a standard to study contaminant interactions with particulate organic matter (Daughney and Fein, 1998). Apart from particulate organic matter, this culture also contained DOM under the form of nutritional compounds or metabolites. The concentrations for DOM were 0, 0.1, 0.4, 0.8, and 1.6 g organic C L^{-1}. The organic matter concentration is indicated with the letter O in the model. Organic matter content was determined according to the Belgian standardization method NBN 357.02. The last standard compound was cholin (Sigma-Aldrich) to mimic the effect of bile salts. This compound was added at concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1, 1.5, and 2.5 g L^{-1}. The bile salt analogue is indicated with the letter B in the model.

The different mixtures were kept at pH 6 with bicarbonate and stirred at 150 rpm for 1 h, after which they were centrifuged. One hundred microliters of the 1500×g and 3000×g supernatants were brought in a 96 multiwell plate and fluorescence was measured with a SpectraMAX Gemini XS spectrophotometer (Molecular Devices, Sunnyvale, CA). The fluorescence was an indication for the amount of PAH analogue present in the supernatant. The fluorescence of the different mixtures gave an idea

how PAHs were complexed by a model bile salt and organic matter and to what extent they remained in the supernatants. Corrections were made for background fluorescence by subtracting the fluorescence intensity from blank sample sets – samples combining the bile salt and organic matter analogues without the fluorescent molecule – from the respective sample sets containing the fluorescent molecule. No significant quenching was observed from the bile salt analogue, nor the organic matter analogue towards the fluorescent molecule. The fluorescence data were used to calculate distribution coefficients, K_d, which give an idea how the PAH analogue is distributed over the different possible complexes that could be present in the mixture. This is explained in more detail in Eq. [3–5] in the Results section.

Evaluation of the mathematical model

We evaluated the utility of the obtained K values to predict PAH release in the batch digestion experiments that were performed on the PAH-contaminated soil samples. The input variables for the model in Eq. [4] were the bile salt (B) and organic matter (O) concentration that were used in the digestions, as well as the free PAH (A) concentrations that were measured. Since observed field data as well as model output had uncertainties, a bivariate scattergram was used to calculate unbiased values for the slope and intercept of the principal axis of the relationship between field data and model predictions (Daughney et al., 2002). An accurate model would yield a unit slope and an intercept of zero, if the predicted PAH release data were plotted against the observed PAH release data from the digests. The coefficient of determination (R^2) was calculated by multiplying the correlation coefficient (R) with the ratio of the variances of the predicted and the observed data.

Statistical analysis

All statistical tests were performed using SPSS 11.0 software (SPSS, 2002). Tests for normality and homogeneity of variances were performed using the Shapiro–Wilkes and Levenes test. Non-normally distributed data were transformed using the natural logarithm for pellet and supernatant PAH amounts and the arcsine transformation on PAH release fractions. Comparison of means was tested with a one-way analysis of variance (ANOVA) using the F test. In case the assumption of equal variances did not hold, the Welch test was used. For supernatant analysis, transformed data were not normally distributed. The nonparametric Kruskal–Wallis test was used on the nontransformed data to compare means. Correlation of release fractions between

digest types were performed using the nonparametric Spearman's rho test. A general significance level of 0.05 for all tests was chosen.

Results

Digestion model

The total PAH concentration in the soil was 49 ± 1.5 mg PAH kg^{-1} soil (Table 3.1) with individual PAH concentrations, except phenanthrene and benzo(a)pyrene, below the Flemish soil cleanup levels for recreational areas. The PAH mass balance recovery for the 12 different digestions (Figure 3.2) ranged from 89 to 108% with an average of 96 ± 6.6%. The PAH input in the different digestion types varied between 752 and 918 µg for 20 g of soil with no PAHs detected in the pancreatic solution or bacterial SHIME suspension. Polycyclic aromatic hydrocarbon release, determined as the PAH fraction present in a 1500×g supernatant, was limited, ranging from 0.4 to 11 µg for the different digest types (Table 3.2), with no significant differences in PAH content between pellets.

Table 3.1 Concentrations for 10 polycyclic aromatic hydrocarbons (PAHs) in the soil samples, as analyzed by gas chromatography–mass spectrometry (GC–MS) compared with the Flemish Soil Remediation Directive. [1]

	CAS nr.	Park soil (mg PAH kg^{-1} DW)	Directive (mg PAH kg^{-1} DW)
Naphthalene	91203	0.27 ± 0.16	160
Phenanthrene	85018	4.88 † ± 0.06	3.5
Fluoranthene	206440	9.53 ± 0.19	40
Benzo(a)anthracene	56553	5.09 ± 0.06	50
Chrysene	218019	6.18 ± 0.43	400
Benzo(b)fluoranthene	205992	6.08 ± 0.30	55
Benzo(k)fluoranthene	207089	3.02 ± 0.09	55
Benzo(a)pyrene	50328	5.88 [2] ± 0.01	1
Indeno(1,2,3-cd)pyrene	193395	4.22 ± 0.87	50
Benzo(ghi)perylene	191242	3.96 ± 0.37	50

[1] Values are means ± standard deviations of four replicates that were taken within 1 m from each other. Sample point coordinates are 51°12'26" N, 3°48'20" E.

[2] Park soil PAH concentration is higher than the Flemish soil remediation directive

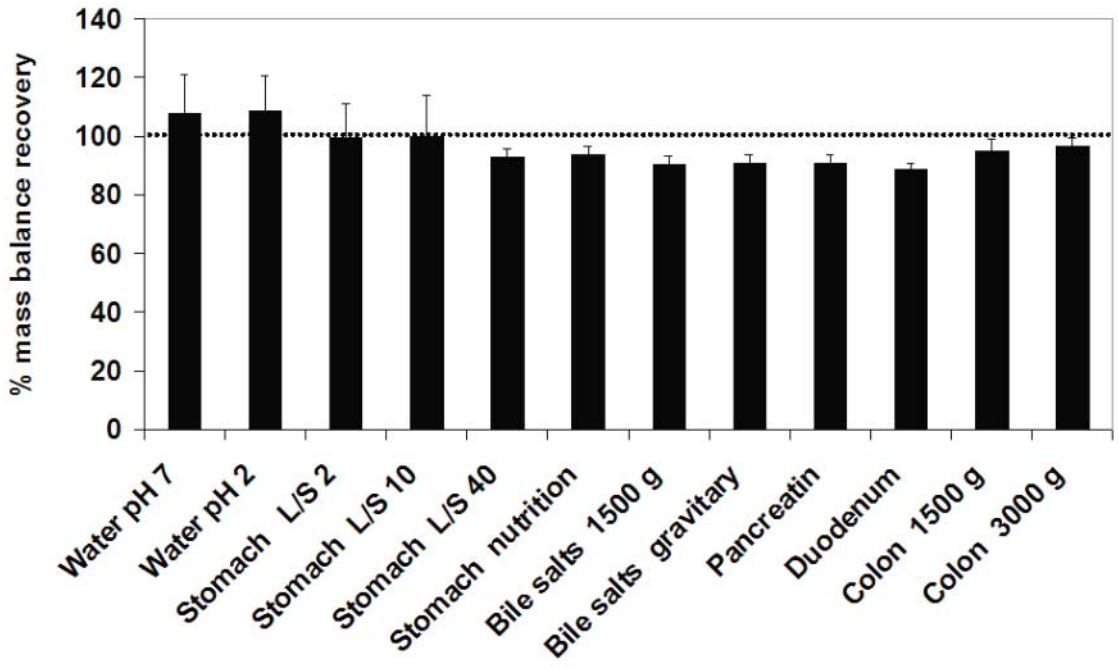

Figure 3.2 Mass recoveries (%) for polycyclic aromatic hydrocarbons (PAHs) in the different soil sample digests. Error bars show standard deviation values of three replicates.

The PAH release was greater ($P < 0.003$) for the water (0.5%) and stomach digestion (0.44%) compared with the release in the duodenum (0.13%) and colon (0.3%) (Table 3.2). Surprisingly, increasing the pH from 2 to 7 had no effect on PAH release whereas increasing the L/S enhanced PAH release. Release of PAHs in digestions with an L/S of 40 was twice as high (0.83%) (Mann–Whitney U test; $P = 0.037$) as that of digests with an L/S of 10 (0.44%) and 16 times as high as that of digests with an L/S of 2 (0.05%). There was a logarithmic correlation between PAH release and L/S (Eq. [1]):

$$\%\text{release} = 0.1454 + 0.2622 \ln(L/S) \qquad R^2 = 1.00 \qquad [1]$$

We postulated that the effect of L/S on PAH release was due to variations in dissolved organic matter. This was confirmed by the fact that addition of food compounds almost doubled PAH release of a stomach L/S 40 digest from 0.83 to 1.4% (Figure 3.3). There was a strong relationship (Eq. [2]) between PAH and dissolved organic matter concentration for all stomach digestions:

$$\text{PAH } (\mu g\ L^{-1}) = 15.35 \times \text{organic C } (g\ L^{-1})\ 0.0486 \qquad R^2 = 0.97 \qquad [2]$$

No relationship was found between dissolved organic matter and PAH release for the colon and the duodenum digests. If the bile digest sample was not centrifuged, but sedimented for 30 min, the PAH content reached the value of 0.43%, which is more than two times greater than that observed during centrifugation (Figure 3.3). Similarly, the PAH content in the colon extract, spun down at 3000×g, was the lowest observed among all digests with a value of 0.10%. Yet, the organic matter content in these duodenum and colon digests was higher than that in the water and stomach digestions (Table 3.2). This suggested that complexes were formed that can spin down at 1500 or 3000 × g if the complexes are large enough.

Table 3.2 Release of PAHs during different stages of digestion. Water, duodenal and colon digests were all performed at a Liquid/Solid ratio of 10, 15 and 25 respectively. Values are averages of n replicates.[1]

	Water pH 7 L/S 10 n = 3	**Water pH 2 L/S 10** n = 3	**Stomach L/S 2** n = 3	**Stomach L/S 10** n = 3	**Stomach L/S 40** n = 2	**Stomach L/S 40** n = 2 **Nutrition**
Centrifugation speed (*g*)	1500	1500	1500	1500	1500	1500
Amount pellet (µg)	917.7	902.7	829.1	844.3	769.9	786.9
Amount supernatant (µg)	4.7	4.3	0.4	3.8	6.3	11.0
Organic matter (g L^{-1})	0.46	-[2]	1.08	2.16	0.99	1.6
Concentration (µg PAH L^{-1})	23.4	21.7	9.0	18.1	7.7	13.4
Release ± s.e. (%)	**0.50 ± 0.026**	**0.48 ± 0.017**	**0.05 ± 0.003**	**0.44 ± 0.11**	**0.83 ± 0.028**	**1.38 ± 0.372**
	Duodenum Bile L/S 10 n = 2	**Duodenum Bile L/S 10** n = 2	**Duodenum pancreatin L/S 10** n = 1	**Duodenum L/S 15** n = 3	**Colon L/S 25** n = 4	**Colon L/S 25** n = 2
Centrifugation speed (*g*)	1500	**1** (sedimented)	1500	1500	1500	**3000**
Amount pellet (µg)	752.9	807.2	723.9	834.1	837.2	805.1
Amount supernatant (µg)	1.4	3.5	1.4	1.0	2.5	0.8
Organic matter (g L^{-1})	0.94	2.35	1.4	1.49	3.05	2.1
Concentration (µg PAH L^{-1})	4.5	11.3	13.5	3.2	6.1	1.9
Release ± s.e. (%)	**0.19 ± 0.067**	**0.43 ± 0.138**	**0.57**	**0.13 ± 0.052**	**0.30 ± 0.015**	**0.10 ± 0.019**

[1] Values are averages of *n* replicates. The term L/S is liquid to solid ratio.

[2] Artefacts in this sample caused an outlier value of 6.31 g L^{-1}.

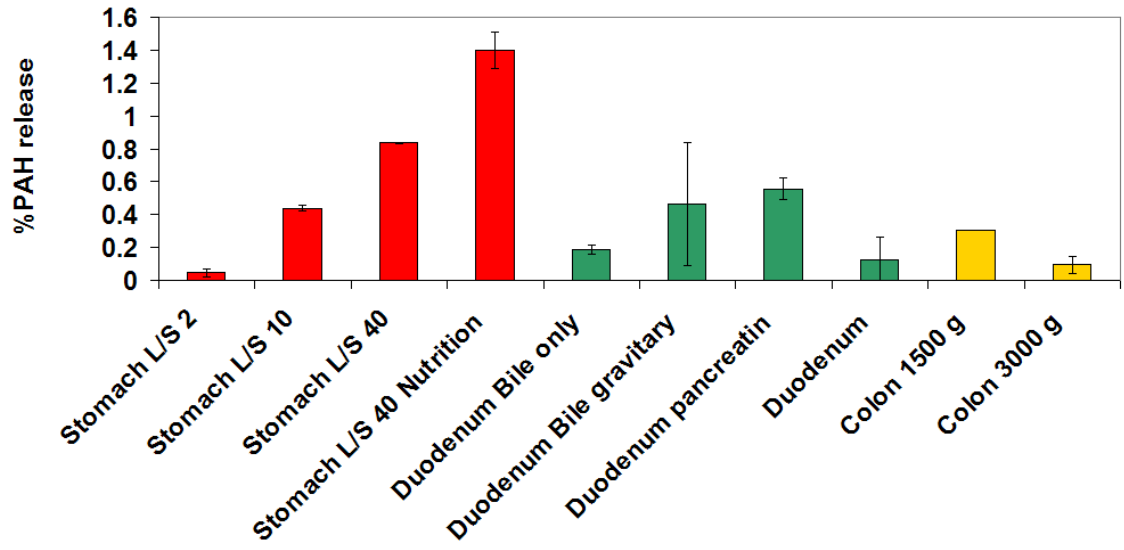

Figure 3.3 Overview of PAH release for the different results within the stomach, duodenum and colon digestions. Values represent averages of 3 or 4 replicates. Error bars display standard error values.

Study of PAH complexation mechanisms

In our attempt to clarify the different complexation mechanisms in the in vitro GI tract, we used three model compounds and mixed them at different concentrations. The three compounds were analogues for low molecular weight PAHs (9-anthracene propionic acid), bile salts (cholin), and organic matter (*Bacillus subtilis* culture). The PAH analogue was fluorescent, which enabled us to rapidly screen for PAHs in the supernatant of the mixtures. We developed a model based on three operational definitions. Polycyclic aromatic hydrocarbons present in the 3000×g supernatant were free PAHs or PAHs in small complexes, PAHs present in the 1500×g supernatant were complexed PAHs in larger complexes, and PAH in the pellet were bound PAHs in large aggregates (Figure 3.4). All three compounds in the mixture could form complexes with each other according to Eq. [3]:

$$A + B + O \leftrightarrow AB + AO_{free} + ABO_{complex} + AO_{complex} + ABO_{bound} + AO_{bound} \quad [3]$$

where: A = PAH concentration

B = bile salts concentration

O = organic matter concentration

From this equation, the overall equilibrium distribution constant can be calculated as follows:

$$K_d = \frac{AB * AO_{free} * AO_{complex} * ABO_{complex} * ABO_{bound} * AO_{bound}}{A^6 * B^3 * O^5} \quad (4)$$

where:

$$AB = K_{AB} * A * B$$
$$AO_{free} = K_{APfree} * A * O$$
$$AO_{complex} = K_{APcomplex} * A * O$$
$$ABO_{complex} = K_{ABPcomplex} * A * B * O$$
$$ABO_{bound} = K_{ABPbound} * A * B * O$$
$$AO_{bound} = K_{APbound} * A * O$$

$$K_d = K_{AB} * K_{AOfree} * K_{AOcomplex} * K_{ABOcomplex} * K_{ABObound} * K_{AObound} \quad (5)$$

The different K values are the equilibrium constants for the respective partial equations. These values were calculated for the mixtures in which two or three model compounds were combined at different concentrations (Table 3.3) The fluorescence analysis returned some variability resulting in K values with rather large variances but fairly constant values over the concentration range tested. The K values were unitless as they were calculated by dividing fluorescence intensity by fluorescence intensity. Multiplying these partial equilibrium constants from Table 3.3 gain yields the overall equilibrium distribution coefficient according to Equation (5). The log value of the overall distribution coefficient K_d as obtained with eq. 4 was -8.3 (sd=2.30), which approached the log K_d value obtained from eq. 5, -7.41 (sd=0.59). The highest partial distribution coefficient, 1.02, was found for the complex between PAH and bile (Table 3.3). Organic matter complexation was also an important factor in PAH complexation with a K value of 0.068. However, tertiary reactions, i.e. the combination of bile salts and organic matter in one complex with PAHs, also contributed substantially with $K_{ABOcomplex}$ values of 0.043 and $K_{ABObound}$ of 0.084.

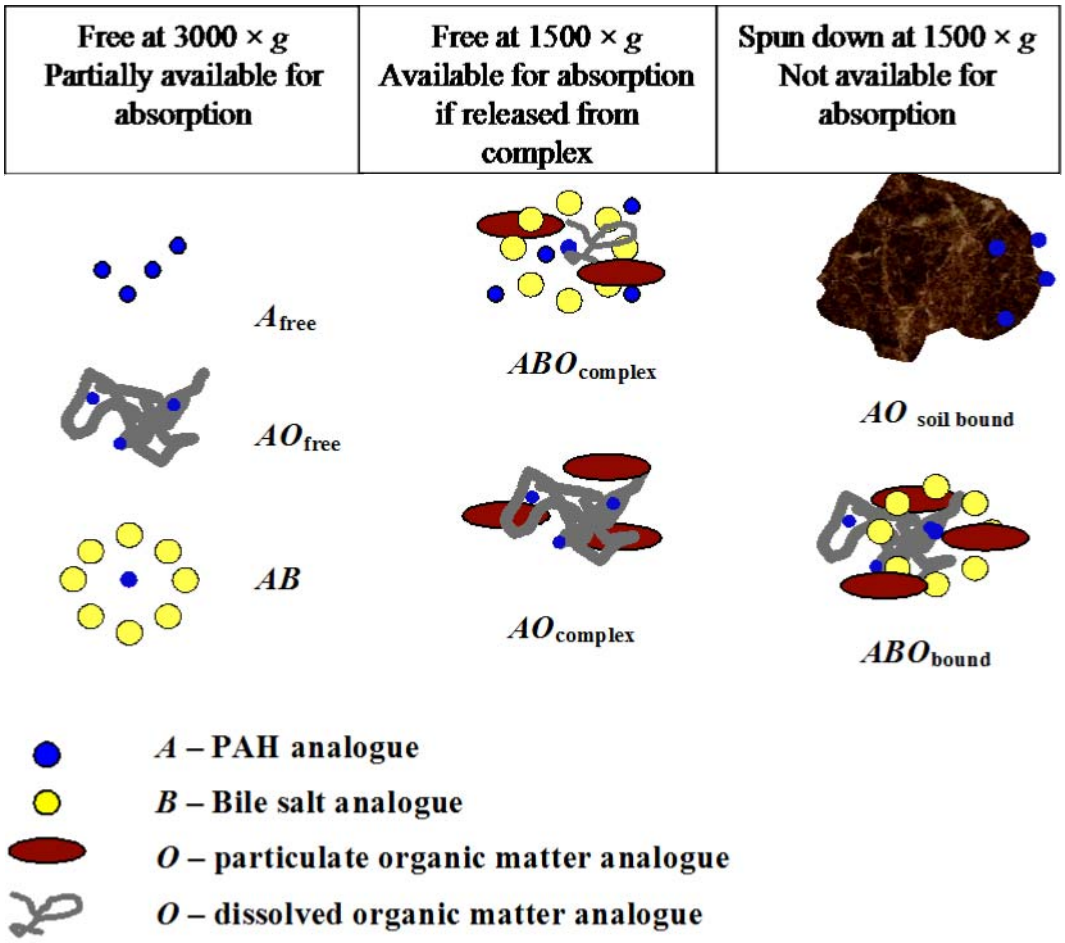

Figure 3.4 Possible complexes that are hypothesized to be formed in the gastrointestinal tract between polycyclic aromatic hydrocarbon (PAH) compounds (A), bile salts (B), and organic matter (O)

Table 3.3 Distribution coefficients for the different partial complexation reactions. [1]

Distribution constant [2]	K_d value [3]	Standard error
$K_{d\ AB}$	1.022	0.0911
$K_{d\ AP\ free}$	0.011	0.0011
$K_{d\ AP\ complex}$	0.068	0.0073
$K_{d\ ABP\ complex}$	0.043	0.0037
$K_{d\ ABP\ bound}$	0.084	0.0089
$K_{d\ AP\ bound}$	0.014	0.0008

[1] Reported values are averages and standard errors from four replicates

[2] Subscripts indicate the different standard compounds, taken up in complexes with A = polycyclic aromatic hydrocarbon (PAH) analogue, B = bile salt analogue, and P = organic matter analogue

[3] Calculated by dividing complex fluorescence intensity by that of the compound in equilibrium

Evaluation of the Mathematical Model

Comparison between Predicted and Observed Data from the Different Digestions. The obtained K values can be compared with each other and give us an idea to what extent complexes between model compounds could be formed. The equilibrium constants, derived from the fluorescence based lab experiments, were used to assess PAH release (predicted values) from the field soil in the different digestion types (observed values). The slope of the predicted versus observed plot was 1.08, while the intercept, -0.61 µg PAH L^{-1}, indicated a slight underestimation of release (Figure 3.5). The majority of the error was accounted for by the model (R^2=0.7937), with only 20% of the variance in observed PAH release being unexplained. We were only able to validate the overall model, but if we assumed that the individual components of the model were also accurate, then the model predictions also gave an indication how PAHs could be allocated in the different complex types. It should be noted that the model only describes how desorbed PAHs are complexed and that it does not take into account PAHs still bound to the soil matrix itself.

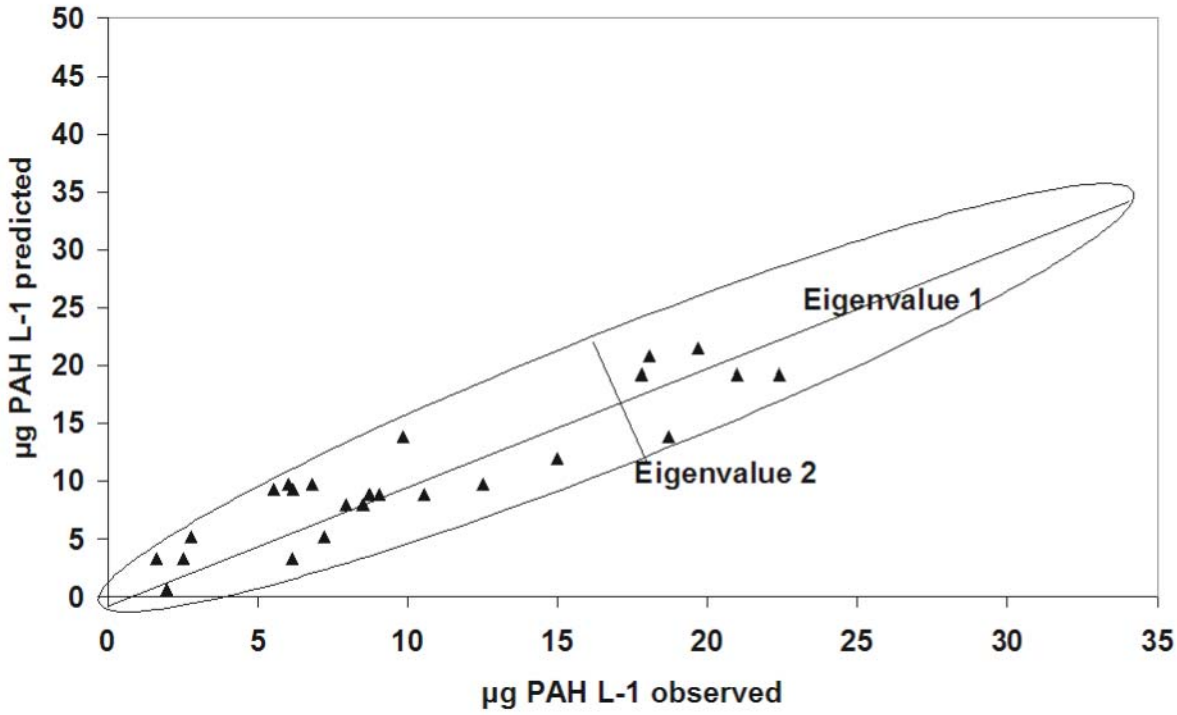

Figure 3.5 Predicted versus observed polycyclic aromatic hydrocarbon (PAH) concentration for the different digest types. The ellipse indicates how the slope and the intercept of the principle axis were calculated using the eigenvalues of the ellipse. The regression coefficient was calculated using the variances of the predicted and observed results as uncertainties were present in both data sets.

For example, in the presence of only pancreatin, 84% of the PAHs in the model remain in a supernatant after centrifugation at 1500×g and this would be divided between complexes (73%) and DOM (11%) (Table 3.4). Fifteen percent of the PAHs would stay behind as bound aggregates in the 1500 × g pellet. In case bile salts alone are added, only 60% of the PAHs would be present in the 1500 × g supernatant, partitioned over micelles (19%), DOM (3%), and complexes suspended in the supernatant (38%), whereas 40% of the PAHs would remain in the pellet. Accordingly, these findings correspond in a proportional way to the PAH amount in the 1500 × g supernatant of the pancreatin (0.57% PAH release) and bile salt (0.19% PAH release) digests (Table 3.2). As a second example, the model predicts that in the colon a much bigger proportion of desorbed PAHs would be present in larger complexes (51%) that spin down at 3000 × g but not at 1500 × g. Only 13% of the released PAHs would remain in the 3000 × g supernatant, divided between bile salts (7%) and DOM (6%). Proportionally, the colon digestion results gave rise to comparable observations to the model predictions, where 0.10% of the PAHs remained in the 3000 × g supernatant, whereas 0.30% of the PAHs were present in the 1500 × g supernatant (Table 3.2).

Table 3.4 PAH distribution among the different complex types, expressed as percentages of the mobilized PAH fraction (typically less than 1%) from a field soil contaminated by 20 years of atmospheric deposition.

Digest type	% in bile salt solution	% associated with dissolved organic matter	% adsorbed to particulates but remaining in supernatant	% adsorbed to particulate that spins down at 1500*g*
Water pH 7	0	11 ± 1.6	73 ± 2.8	15 ± 2.3
Water pH 2	0	11 ± 3.6	73 ± 6.3	15 ± 5.1
Stomach L/S 2	0	11 ± 1.6	73 ± 2.8	15 ± 2.3
Stomach L/S 10	0	11 ± 3.3	73 ± 5.6	15 ± 4.6
Stomach L/S 40	0	11 ± 1.5	73 ± 2.7	15 ± 2.1
Stomach nutrition	0	11 ± 2.4	73 ± 4.1	15 ± 3.4
Bile salts	19 ± 1	3 ± 0.9	38 ± 1.4	40 ± 1.5
Pancreatin	0	11 ± 2.1	73 ± 3.6	15 ± 4.0
Duodenum	19 ± 0.7	3 ± 0.6	38 ± 1.0	40 ± 1.1
Colon	7 ± 1.3	6 ± 1.3	51 ± 2.1	36 ± 1.8

Release of individual PAH compounds

No significant differences in release were observed between different digestive compartments for individual PAHs (Figure 3.6), except for higher naphthalene release in water (p=0.025) and colon (p=0.039) digests. However, if the PAHs are grouped into high (>0.012 mg L^{-1}) and low (<0.012 mg L^{-1}) solubility groups, the low-solubility PAHs were mobilized to a greater extent than would be expected from their solubility. Logically, PAH release would decrease with decreasing solubility, which was indeed the case from naphthalene to benzo(a)anthracene. The individual PAH release, however, increased again with decreasing solubility from chrysene on to the most hydrophobic PAHs, like benzo(ghi)perylene. Other common molecular descriptors such as hydrogen to carbon ratios, molecular weight, molecular surface area, and log octanol–water partitioning coefficient (K_{ow}) values of the individual PAHs were evaluated using Microsoft Excel (Microsoft, 2002) for their possible relationship to the release of individual PAH compounds in the water, stomach, duodenum, and colon digest. The most accurate parameter showing a correlation with the release rates of different individual PAH compounds was, however, the logarithm of the respective PAH solubility values (Eq. (6): S = solubility in mg L^{-1}):

$$\% \text{ release} = 0.368 + 0.206 \log S + 0.0675 (\log S)^2 \qquad R^2 = 0.92 \qquad (6)$$

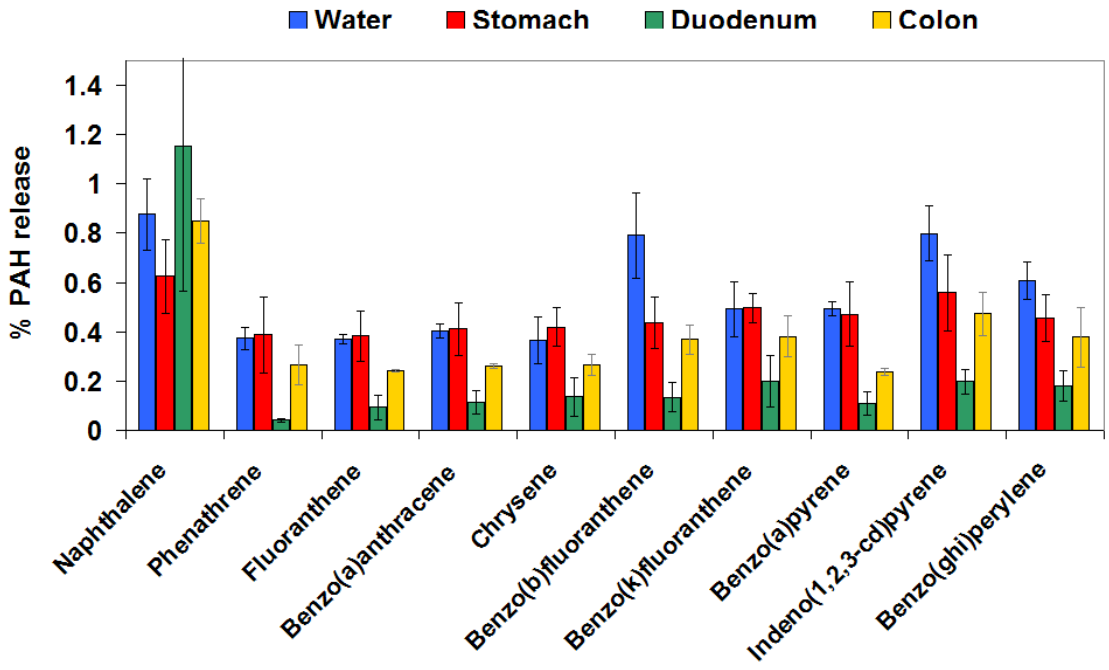

Figure 3.6 Release percentages of individual polycyclic aromatic hydrocarbons (PAHs) into the aqueous phase during water, stomach, duodenum, and colon digestion. Results plotted are averages. Error bars show standard error values from three replicates.

Discussion

The combination of digestion experiments and the development of a predictive model in this research gave more insight on PAH mobilization from a soil matrix in the human digestive tract. Contaminant mobilization from a soil matrix and subsequently complex formation in the gastrointestinal tract are crucial first steps in the assessment of oral bioavailability, yet the understanding of these processes is incomplete.

During intestinal digestion, PAHs desorb from the soil matrix and they can be divided into three groups. A first group comprises bound PAHs, mobilized from the soil, but adsorbed to large aggregates that spin down at 1500×g. A second group embodies complexed PAHs with bile salts and/or organic matter, which stay suspended after a 1500×g centrifugation step, but spin down at 3000×g. Finally, the third group remains in solution after centrifugation at 3000×g and consists of free PAHs and PAHs taken up in small complexes with bile salts or dissolved organic matter. The choice of distinguishing these three groups merely relies on providing more insight on contaminant mobilization and complexation processes in the gut. They are not based on physiologically relevant values. However, the latter group, comprising the free PAHs and PAHs in small complexes, contains the bioaccessible fraction, which is the fraction available for intestinal absorption. This is an important fraction as it preceeds oral bioavailability. In their *in vitro* study, Oomen et al. (2001) used 3000×g to assess bioaccessible lindane and PCB concentrations. However, Holman et al. (2002) considered complexes smaller than 0.5 µm as bioaccessible, as these are the only ones able to pass between the microvilli.

The complexation phenomena are not merely a prerequisite to uptake by blood or lymph, but are the dominant factors that influence the PAH fraction that can be taken up through the small intestinal wall. Understanding the distribution of PAHs in different complexes along the human GI tract may explain the differential toxicity observed for ingested PAHs. For example, five- or six-ring PAHs are better inducers of arylhydrocarbon (AhR) biotransformation enzymes in the duodenum, whereas three- or four-ring PAHs are more prone to induce AhR in the liver (Hrudey and Rousseaux, 1996). Our model successfully predicted PAH release during passage through the SHIME but neither the SHIME nor any other gastric simulator is currently able to mimic the highly dynamic and active gut epithelial.

Digestions

This study indicated that PAH ingestion from contaminated soil may be hazardous. Polycyclic aromatic hydrocarbon mobilization from a low-contamination soil yielded a PAH concentration in the chyme solution ranging from 2 to 23 µg L^{-1}, which is 20 to 200 times greater than the European drinking water directive of 0.1 µg L^{-1}, although the latter is not always derived out of toxicological consideration. This occurred at L/S between 10 and 40, which are lower values compared with the ratios of 60 to 1000 used in previous studies (Hack and Selenka, 1996; Holman et al., 2002; Oomen et al., 2000), but comparable with the L/S experienced by children during fasting conditions. These L/S are derived from a hypothetical soil ingestion of 20 g, a chyme production of 40 mL h^{-1}, and a residence time of 8 h during the passage through stomach and small intestine. During fasting conditions, the chyme production drops to 40 mL h^{-1}, whereas 250 mL h^{-1} is produced while eating (Paterson et al., 2000). An acute exposure to 20 g soil d^{-1} would yield an L/S of 16 while fasting and 100 while eating. Our data are derived from very high ingestion rates and the high concentrations indicate that ingestion of soils, even at low contamination levels, may be hazardous for children as a risk group of high concern. The influence of L/S on contaminant release has thus far only been investigated in vitro to mimic ingestion of metal-contaminated soils by adults, covering an L/S range of 100 to 5000 (Hamel et al., 1998). Only a slight influence of L/S on metal release was observed. Although the L/S of 2 from this research was not derived from physiological data, we considered this data point together with the L/S 10 and L/S 40 stomach digests to study the influence of L/S on PAH release at L/S ranges below 100. Concerning the level of soil contamination as such, this should not be underestimated as indicated by an atmospheric deposition of PCBs or PAHs up to 20 µg m^{-2} d^{-1} in an urban area (Sanders et al., 1992, 1993). This contamination route was considered in the recent SOWA project (Halm and Grathwohl, 2003) and indicates that ingestion of soils, especially by children, is a relevant issue to investigate.

The release percentage of the PAH compounds from the soil matrix was low, 0.1 to 1.4%, and can be compared with the 0.5 to 2% GI solubility that Holman et al. (2002) obtained with an in vitro fasting digestion of crude-oil-contaminated soils. Kögel-Knabner et al. (2000) assessed the aqueous mobilization of PAHs in aged soils and got similar release values of 1 to 3%. They found the mobilization process was highly dependent on organic matter content and ionic strength of the aqueous solution. These values are much lower than those observed by others (10–60%), investigating release of hydrophobic organic contaminants during in vitro digestion of freshly contaminated soils or Organisation for Economic CoOperation and Development soils (Hack and

Selenka, 1996; Oomen et al., 2000). Our results are derived from a historically contaminated soil, which may explain this descrepancy due to possible aging effects.

Polycyclic aromatic hydrocarbon release, identified as the fraction present in the 1500 × g supernatant of the digests, changed significantly as digestion proceeded from stomach to large intestine. The parameter that influenced this release the most was the organic matter content in the aqueous phase. Organic matter provides extra complexation sites for contaminant adsorption. This same mechanism was described in a study of Oomen et al. (2002) and had previously been proposed by Hack and Selenka (1996). However, this trend was not observed in the duodenum and colon digestions, where higher organic matter content did not lead to higher PAH release. This necessitated the development of a more complex model.

Mathematical Model

We developed a model with which the interactions of analogues for PAHs, bile salts, and organic matter were studied. The obtained equilibrium constants (K values) could be compared with one another and allowed us to gain more insight to what extent and in what complex types PAHs were present in a modeled intestinal suspension. These distribution constants thus have no direct physical significance that could be used for other contaminants or other soil types.

In our experiments, we found that bile salts and dissolved organic matter interacted during the mobilization process of soil-bound PAHs. This is supported by Oomen et al. (2000), who found higher lindane and PCB mobilization rates in a 3000×g supernatant at higher bile salt and protein concentrations. They also used several mixtures of these compounds to calculate the distribution coefficients for the formation of PCB complexes. This method worked for the free PCB fraction present in the 3000×g supernatant. However, it did not incorporate the formation of larger complexes combining bile salts and dissolved or particulate organic matter, typically encountered in the large intestine. By including and calculating the formation of these intermediate complexes, the mathematical model accurately (8% overestimation) and closely (R^2 = 0.8) predicted observed PAH concentrations released from soil containing aged PAHs. By simplifying the complicated intestinal suspension, using three standard components, our model could not only predict PAH release from a field situation, but it also gave an idea about the types of complexes being formed. Normally, PAHs as free compounds or complexed in bile salt micelles can be taken up through the gut wall. Which fraction of PAH complexes is absorbed through the intestinal wall depends on the complex size. As the small intestine also contains some Peyer's Patch cells able to

take up particles of a few micrometers in size, PAHs in small complexes can be taken up in a small amount through the small intestinal wall. Thus, the amount released and also the type of complex formed are important for the toxicity of released PAHs. We were able to directly evaluate the accuracy of the model for PAH release but we lack direct spectroscopic data to confirm PAH complexation in the human gut.

Differences in PAH compound

Although empirically obtained, differences in release between PAHs were best described by a quadratic model (Eq. [6]). For the low molecular weight PAHs, a decrease in release was observed with decreasing solubility, whereas the release in high molecular weight PAHs increased with decreasing solubility. While this trend can be modeled using a quadratic response function, this trend might have been obtained due to measurement errors. However, what is clear is that PAH release does not continue to decrease in concert with aqueous solubility. The rationale for this was conceptualized by MacKay and Gschwend (2001), who described two competing factors influencing PAH contaminant release. They investigated the PAH concentrations in the ground water of a coal tar site and found that organic colloids enhanced PAH solubility by a factor of 16 for benzo(a)pyrene and up to 50 times for indeno(ghi)perylene. The enhancing solubilization effect by colloids increased with increasing hydrophobicity of the compound and may explain the increased release of high molecular weight PAHs we observed in the in vitro intestinal suspension. Such an increase in solubility for the high molecular weight PAHs is worrying for risk assessers as many of these compounds are known to possess carcinogenic or even estrogenic effects as they get bioactivated on intestinal absorption (Roos et al., 1996; Roos, 2002).

Conclusions

In this study, we showed the importance of simulating the gastrointestinal tract of high risk groups that children form in risk assessment. As soil ingestion parameters like L/S differ substantially from other digestion models for other risk groups, the outcome of this study shows that contaminant concentrations in the intestinal tract are higher for higher L/S, and that the total amount of released contaminant will be substantial even when the soil is only slightly contaminated. Bile salts and organic matter in the digest aqueous phase have a dual effect on contaminant mobilization. On the one hand, they increase desorption as hydrophobic contaminants can more easily complex with these

compounds in the aqueous phase. On the other hand, complexes may be formed that decrease the chance of actually being absorbed through the small intestinal wall. This complicated complexation process was predicted by a mathematical model we developed, based on standard analogues for PAHs, bile salts, and organic matter. The model was useful in this specific case of a PAH-contaminated soil and could predict how many PAHs were released in the GI tract during in vitro digestion. Additionally, it allowed us to gain more insight into the complexation phenomena that are crucial processes in the gut lumen before bioaccessibility and oral bioavailability. An aspect of this research that requires further attention is the increased solubility for the high molecular weight PAHs, as these compounds are known for their possible carcinogenic or estrogenic properties. The combination of bioassays and analysis of PAH metabolites might deliver substantial information in the ongoing research.

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CHAPTER 4

Liquid chromatography–mass spectrometry analysis of hydroxylated polycyclic aromatic hydrocarbons, formed in a simulator of the human gastrointestinal tract

Abstract

Described is a liquid chromatography mass spectrometry (LC-MS) procedure for the determination of hydroxylated biotransformation products of poycyclic aromatic hydrocarbons (PAH) in the human gastrointestinal tract. The formation of hydroxylated PAHs was monitored upon incubation of PAHs with colon microbiota from the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The analytical method consisted of a biomass removal step followed by a solid phase extraction step using C18 packed columns to remove non-digested food compounds and microbial metabolites that interfere with the detection of the target compounds. For quantification, 9-hydroxy phenanthrene $^{13}C_6$ was used as the internal standard. The detection limits of the hydroxylated PAHs were generally in the range 0.36 to 14.09 µg L^{-1}, based on a signal/noise ratio of 3:1. The recovery of hydroxylated PAHs in intestinal suspension was variable ranging from 45% to 107%, with relative standard deviation between 5% and 17%. The analytical procedure was used to show the microbial production of 1-hydroxypyrene and 7-hydroxybenzo(a)pyrene, metabolites that may give colon incubated PAHs bioactive properties.

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Introduction

The levels of polycyclic aromatic hydrocarbons (PAH) in the environment has remained an area of extensive study in modern times, largely because of their prevalence in the environment and the mutagenic, carcinogenic and estrogenic effects of their metabolites (Mastrangelo et al., 1996; Szeliga and Dipple, 1998; Hirose et al., 2001; Mersch-Sunderman et al., 2001). For urban areas in particular, nearby industry and dense traffic may lead to atmospheric deposition of PAHs at levels up to 20 μg m^{-2} per day (Sanders et al., 1993). Inhalation of PAH containing particulates and ingestion of contaminated food are important exposure routes to the human body (Becher et al., 1984; Buckley et al., 1992). PAH accumulation in the upper soil layers may also pose a serious risk to public health through for example, possible ingestion of contaminated soils or badly cleaned vegetables from these soils. There is growing interest in the study of hydroxylated PAH metabolites, as important intermediates of PAH biotransformation processes in the human body (Jongeneelen, 1996). Monitoring of urinary or biliary 1-hydroxypyrene and other PAH metabolites is often used as biomarkers for PAH exposure in aquatic and terrestrial ecosystems, but also for exposure to humans (Strickland et al., 1996; Adonis et al., 2003). Apart from their importance as transformation products, hydroxylated PAHs may possess estrogenic properties (Hirose et al., 1998; Fertuck et al., 2001a) and some are related to mutagenic and carcinogenic effects.

Analysis of hydroxylated PAHs and PAH metabolites in general is usually performed using high-performance liquid chromatography with fluorescence detection (HPLC-F) (Escartin and Porte, 1999; Ruddock et al., 2003; Stroomberg et al., 2003). Earlier studies of PAH biotransformation have also applied gas chromatography (GC) coupled to mass spectrometry (MS) with chemical ionization to screen for PAH hydroxylates, while the PAH parent compounds were analyzed through classical HPLC with diode array detection (DAD) (Cajthaml et al., 2002). The liquid chromatography–mass spectrometry (LC–MS) methods have utilized atmospheric pressure chemical ionization or electrospray ionization (ESI) in both positive and negative ionization mode to screen for hydroxylated PAH compounds (Galceran and Moyano, 1994; Galceran and Moyano, 1996). However reported detection limits of around 0.5 μg mL^{-1} are relatively high when taking into account that concentrations in the range 0.039–2.5 μg mL^{-1} have been previously used for detecting in vitro mutagenic effects and that urinary 1-hydroxypyrene levels of only 2.5 ng mL^{-1} may occur (Hauser et al., 1997; Adonis et al., 2003). Thus far, most research on PAH exposure and detection of

metabolites has been performed by analyzing biliary or urinary samples. Little is known however, about the biotransformation processes of environmental contaminants by intestinal microbiota from the gastrointestinal tract. This is due in part to a need for analytical methods that aresuitable for determining the transformation of xenobiotic compounds in the microbe and enzyme diverse environment of the colon (Ilett et al., 1990). In this study, we present a LC–ESI–MS methodology for the quantification of hydroxylated PAH metabolites and some selected PAHs in water and the human gastrointestinal tract. The detection of these microbially formed hydroxylated PAHs in intestinal suspension has not been described previously.

Materials and Methods

Reagents

The PAH hydroxylates (Figure 4.1) investigated were 1-hydroxynaphthalene (1OHN), 1-hydroxypyrene (1OHP), 9-hydroxyfluorene (9OHF) and 9-hydroxyphenanthrene (9OHPh), obtained from Sigma-Aldrich (Bornem, Belgium) and 7-hydroxybenzo(a)pyrene (7OHBaP), 7,8-dihydroxybenzo(a)pyrene (78OHBaP), and 4,5-dihydroxybenzo(a)pyrene (45OHBaP) obtained from the NCI Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO, USA). One additional compound, 2-phenylphenol (2PP) (Sigma-Aldrich, Bornem, Belgium), was also investigated as a putative PAH metabolite. PAH parent compounds were obtained from Sigma-Aldrich (Belgium), Janssen Chimica (Geel, Belgium) and Supelco (Oakville, Canada). Stock solutions of the PAH hydroxylates were prepared in methanol and contained 100 μg mL^{-1} 1OHN, 90 μg mL^{-1} 1OHP, 93 μg mL^{-1} OHF, 98 μg mL^{-1} 9OHPh, 124 μg mL^{-1} 2PP, 8 μg mL^{-1} 7OHBaP, 16 μg mL^{-1} 45OHBaP and 11 μg mL^{-1} 78OHBaP. A series of working standards was prepared by diluting the stock solution with 50% methanol to final concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.01 μg mL^{-1} for each individual PAH hydroxylate. In addition, similar standards were prepared with centrifuged colon suspension as diluent to compensate for possible matrix effects in the quantitative LC-ESI-MS analysis of colon suspension samples.

Stock solutions of the corresponding PAH parent compounds were made in acetonitrile with concentrations of 218 μg mL^{-1} naphthalene, 220 μg mL^{-1} pyrene, 212 μg mL^{-1} fluorene, 196 μg mL^{-1} phenanthrene and 164 μg mL^{-1} benzo(a)pyrene. To determine the removal of other PAH components during gastrointestinal digestion, an additional set of stock solutions containing 252 μg mL^{-1} acenaphthylene, 180 μg mL^{-1}

anthracene, 180 µg mL^{-1} fluoranthene, 164 µg mL^{-1} benzo(a)anthracene and 168 µg mL^{-1} chrysene were used. No hydroxylated derivatives from these latter PAHs were analyzed. Mixed standard dilution series were prepared by diluting the stock solution with 50% methanol to final concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.01 µg mL^{-1} for each individual PAH compound. In addition, similar standards were prepared using blank colon suspension as the diluent. These solutions were used for LC-DAD analysis.

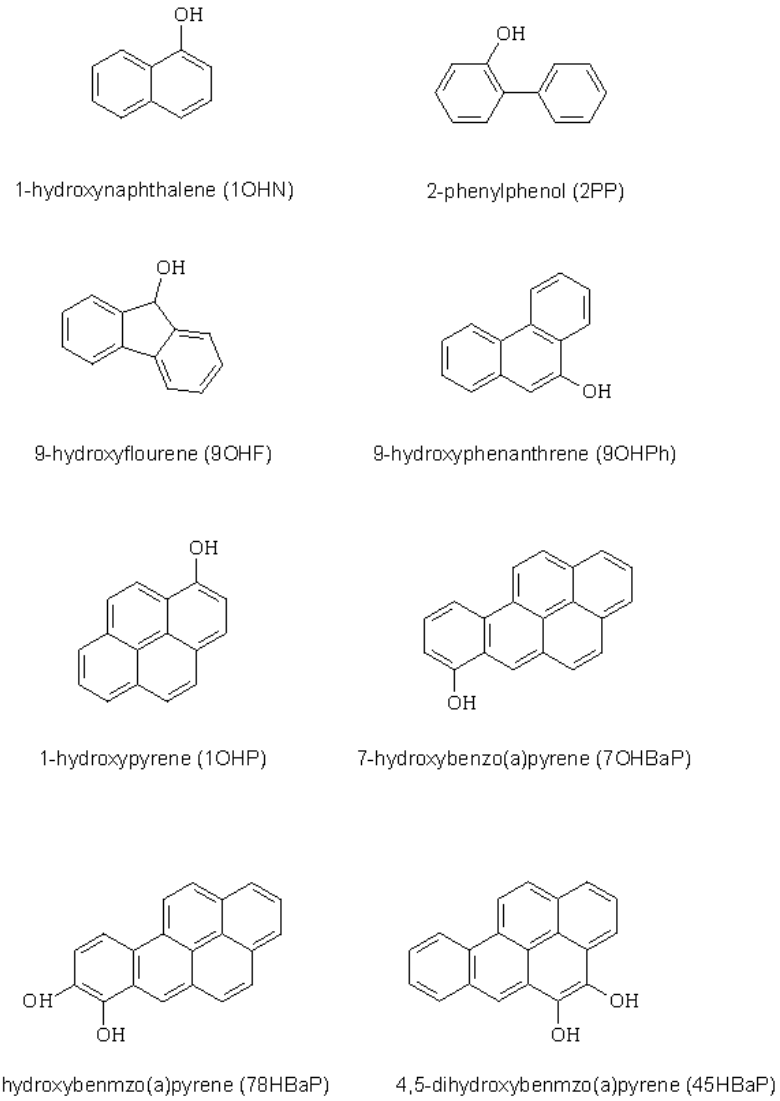

Figure 4.1 Chemical structures of PAH hydroxylates

Incubations

Samples were taken from the colon vessels of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This dynamic model of the human gastrointestinal tract consists of 5 compartments representing the stomach, duodenum, colon ascendens, colon transversum and colon descendens, respectively (http://www.avecom.be/shime_i.html). The colon suspension contains an *in vitro* cultured microbiota that is isolated from human feces and is representative for the *in vivo* colon microbial ecology (Molly et al., 1993). The organic matter content of colon suspension typically varies around 2.2 g L^{-1} and originates from some food compounds but mainly microbiota at concentrations of 11 log CFU mL^{-1}.

A volume of 500 mL of colon suspension was sampled from the SHIME reactor and distributed in aliquots of 50 mL to ten penicillin flasks. Each flask contained a different PAH compound. To avoid solubility problems, PAHs were first dissolved in ethanol and then introduced separately into the respective flasks at final concentrations for naphthalene, 2.56 µg mL^{-1}, acenaphtylene, 3.04 µg mL^{-1}, fluorene, 3.32 µg mL^{-1}, phenanthrene, 3.56 µg mL^{-1}, anthracene, 3.88 µg mL^{-1}, pyrene, 4.04 µg mL^{-1}, fluoranthene, 4.05 µg mL^{-1}, benzo(a)anthracene, 4.56 µg mL^{-1}, chrysene, 4.56 µg mL^{-1} or benzo(a)pyrene, 5.04 µg mL^{-1}. These suspensions were then incubated for 24 hours at 37°C. After this colon incubation, samples were centrifuged at 3000×*g* for a duration of 10 min. to remove biomass and subsequently stored at -20°C prior to analysis for PAH hydroxylates and parent compounds.

In order to assess the extent of bacterial degradation, a number of control samples were included in the experimental setup. Firstly, in order to determine whether hydroxylated PAHs could be formed by extracellular enzymes, PAHs were incubated in colon suspension that had been centrifuged (3000×*g*, 10 min) to remove microbial biomass. Secondly, un-dosed colon samples were analysed to serve as a negative control as they presumably do not contain any of the PAHs. Thirdly, a stomach and small intestine digest of the PAHs prior to the colon incubation was performed as previously described in Chapter 3 and analyzed (Van de Wiele et al., 2004a). These digestion steps contained no bacteria, thus, no biotransformed PAHs should be measured from these samples.

Samples were also incubated in the presence of β-glucuronidase and aryl sulfatase, both obtained from Sigma-Adrich (Belgium). After the PAH parent compounds had been incubated in SHIME suspension, a 1 mL aliquot of these samples were diluted in 1 mL 0.1 M acetate buffer and the pH was adjusted to 5 with sodium hydroxide. A volume of 400 µl β-glucuronidase (100 U mL^{-1}) and 250 µl aryl

sulfatase (60 U mL^{-1}) were added and the mixture was incubated for 6 hours at 37°C to hydrolyze the PAH conjugates.

Sample preparation

Due to the complexity of the colon suspension from the SHIME reactor, matrix interference was anticipated. To assess the extent of such interfences, calibration standards were prepared in both Milli-Q® (MQ) water and centrifuged colon matrix. Standard curves and recoveries in MQ water and colon matrix were compared to determine the influence of the colon matrix on the detection and quantification of the PAH hydroxylates and parent compounds. All samples were thawed and subsequently subjected to a solid phase extraction (SPE) using PrepSep™ C18 (250 mg) (Fisher Scientific, Edmonton, Canada). The C18 columns were placed on top of a SPE vacuum manifold (Chromatographic Specialty, Ontario, Canada) to aid solvent elution through the column. The C18 packing material was first conditioned with 10 mL of methanol and subsequently rinsed with 10 mL water. Sample volumes of 5mL were loaded on the columns; the aqueous solution was eluted as waste using a gentle vacuum together with 10 mL of MQ water to remove hydrophilic impurities that were present within the complex colon matrix. For the mixed standard solutions, the analytes (PAHs and hydroxylates) were eluted together with other hydrophobic compounds by loading four times 2.5 mL of methanol on the column. During the method development phase, each eluent was analyzed separately to determine in which fraction a given analyte was recovered. As no significant amounts of analytes eluted in the fourth fraction, all other samples were eluted with 7.5 mL of methanol. Aliquots of 1.0 mL of the sample extracts were subsampled into amber glass vials and stored at 4°C prior to LC-ESI-MS analysis.

Instrumental conditions

HPLC analysis was performed using a Waters 2695 (Milford, MA, USA) separation module. The HPLC pump was primed with fresh eluent on a daily basis. The selected column was a 2.1 mm × 100 mm, 3.5 µm particle size, Waters XTerra MS C18 column (Milford, MA, USA) which was kept at a constant temperature of 26 °C. The binary eluent system consisted of methanol:water 90:10 v/v (eluent A) and methanol:water 10:90 v/v (eluent B). Gradient elution was performed using 50% eluent A for 5 min, then a linear gradient from 50% to 95% eluent A for 30 min at a flow-rate of 200 µl min^{-1}. At the end of each cycle, 95% eluent A conditions were held for 5 min to ensure all

sample components were eluted from the column. An injection volume of 10 µl was utilized employing a Waters 2695 autosampler for both samples and calibration standards. A diode array UV detector was plumbed inline prior to the mass spectrometer for detection of the parent PAHs at a wavelength of 280 nm.

Mass spectrometry analysis was performed with a Quattro Ultima Mass spectrometer (Micromass Technologies, Manchester, UK) that was equiped with an electrospray interface operating in the negative ion mode. Instrumental control and data acquisition was performed with MassLynx software version 3.5. The ESI source was operated at 90°C, desolvation temperature 200°C, cone voltage 61 V, and a capillary voltage of 2.74 kV. Nitrogen gas served as the cone gas (flow rate of 159 L h^{-1}), desolvation gas (490 L h^{-1}) and nebulizer gas (set to maximum). The detector multiplier voltage was set to 650V. Selected ion monitoring was employed for quantitative analysis monitoring the $(M-H)^-$ of m/z 143.2 for 1OHN, 169.2 for 2PP, 181.2 for 9OHF, 193.2 for 9OHPh, 199.2 for 9-hydroxyphenanthrene $^{13}C_6$ (internal standard), 217.2 for 1OHP, 267.2 for 7OHBaP and 285.2 for 4,5OHBaP and 7,8OHBaP. The dwell-times were set at 0.3 s and the inter-scan delay time was 0.1 s. As electrospray is a soft ionization technique, only the $(M-H)^-$ were formed. As it is preferred to monitor more than one ion per component of interest for quantification and confirmation purposes, cone induced and collision induced dissociation were evaluated. Under various experimental conditions the molecular ions did not form product ions therefore confirmation using a secondary SIM channel or reaction monitoring was not possible. In lieu of qualifier ions, the in-line diode array detector was used as a secondary confirmation of the identification of the hydroxylated PAHs. Detection of possible unknown metabolites of the PAH parent compounds was also performed with the mass spectrometer operated in full scan mode over a range of 100 to 450 m/z.

Results and discussion

Calibration and matrix effects

Detection of hydroxylated PAHs in an intestinal suspension has not been described before. The difficulty of analysis of intestinal suspension pertains to the complexity of the matrix. The colon matrix is comprised of non-digested food components, several excretion products and hundreds of microbial metabolites. Following sample clean-up, several hydrophobic compounds may still co-elute with the target analytes and thus interfere with the identification and quantification of the PAH hydroxylates during LC-ESI-MS analysis. In order to compensate for matrix effects on both chromatographic separation and electrospray ionization suppression/enhancement, a centrifuged blank colon suspension (3000×g, 10 min.) was used as the diluent when preparing the calibration standards. Calibration curves were therefore independently prepared, using either MilliQ® water or colon suspension that had been spiked with PAH standards and their hydroxylated derivatives at concentrations of 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg L^{-1}. For most of the compounds, linear correlations (R^2>0.99) between peak areas and analyte concentrations were obtained. The calibration curve for 45OHBaP and 78OHBaP showed a lower correlation (R^2=0.947), whereas quadratic calibration curves were obtained for pyrene (R^2=0.999) and fluoranthene (R^2=0.998).

Under the experimental conditions utilized for the gradient and column, the majority of the analytes were base-line resolved from interfering components. Representative ion chromatograms for the hydroxylated PAHs respectively for (a) concentrations of 1 mg L^{-1}, spiked in colon suspension; and (b) blank colon suspension with no spike addition of the target analytes are given in Figures 4.2 and 4.3. There was little to no background signals from the colon matrix that interfered with the detection of the selected PAH hydroxylates, nor with the detection of the target PAH parent compounds when spiked at 1 mg L^{-1} in colon suspension (Figure 4.4). However, the separation of 45OHBaP and 78OHBaP was compromised compared to that obtained from MQ water (data not shown). This loss of chromatographic resolution was probably due to column overloading from the complex sample matrix. The two respective analytes were therefore quantified together as a pair in colon suspensions. Future method modifications should incorporate the improvement of HPLC separation in order to report individual levels of these two compounds in the complex colon suspension.

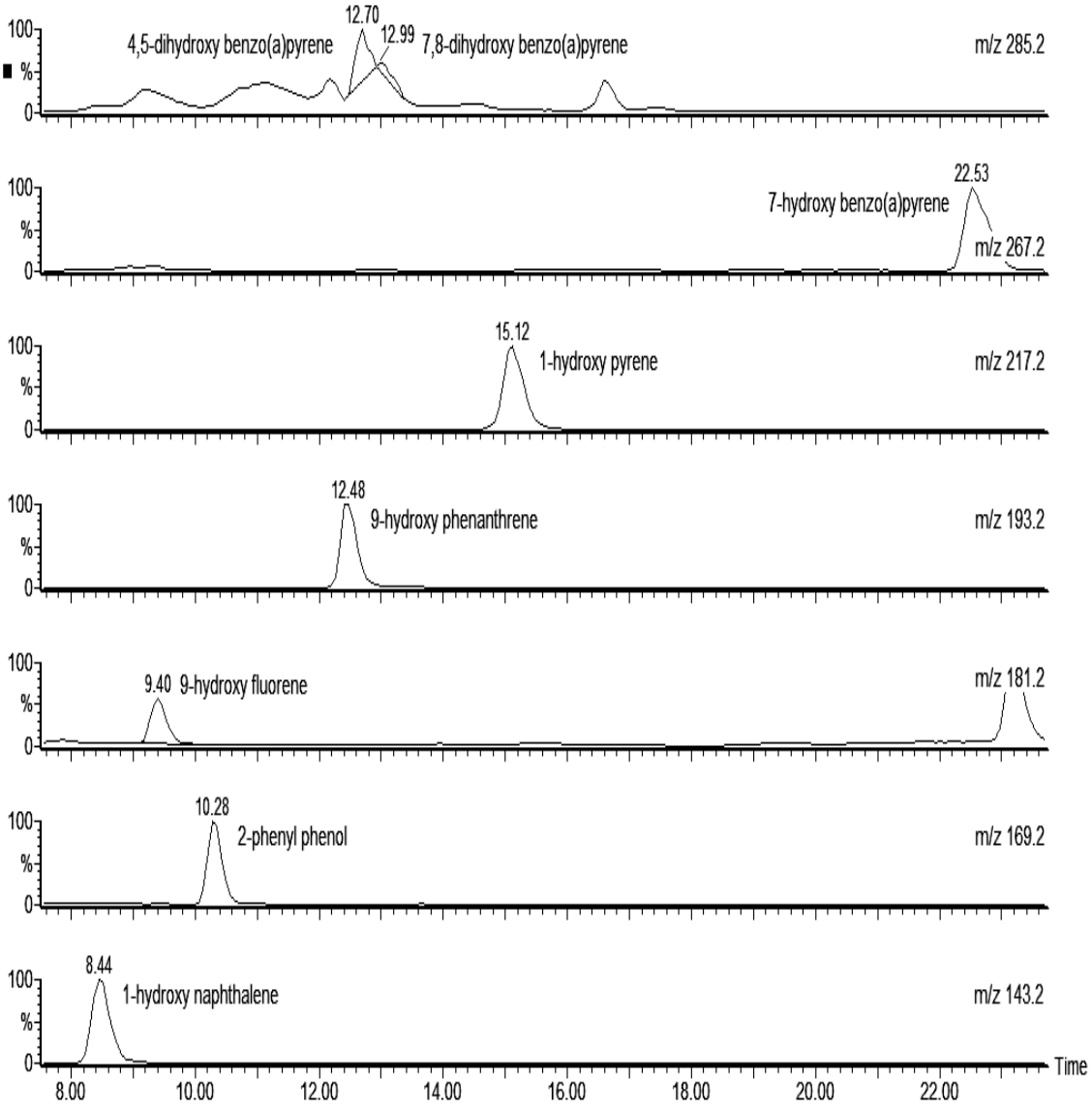

Figure 4.2 LC-MS ion chromatogram of the PAH hydroxylates fortified in colon suspension at a 1 mg L^{-1} concentration. Retention time is expressed in minutes.

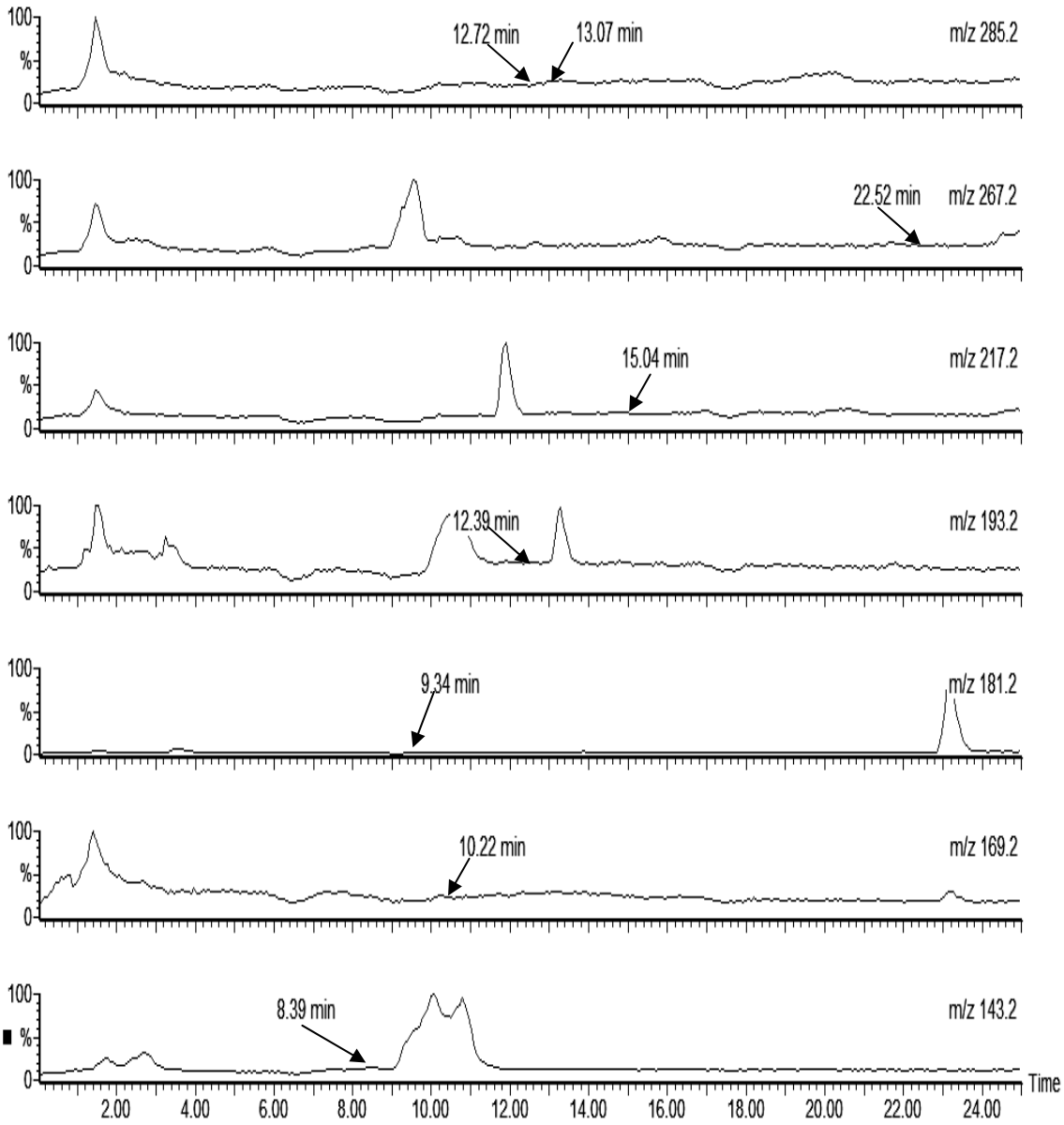

Figure 4.3 Chromatogram for m/z ratios of PAH hydroxylates in a blank sample, which is typically colon suspension to which no PAHs or hydroxylated PAHs were supplemented. Time is expressed in min. Background signals from the colon suspension matrix eluted at retention times other than those from the standards of the PAH hydroxylates. Hence, little to no matrix interference was expected.

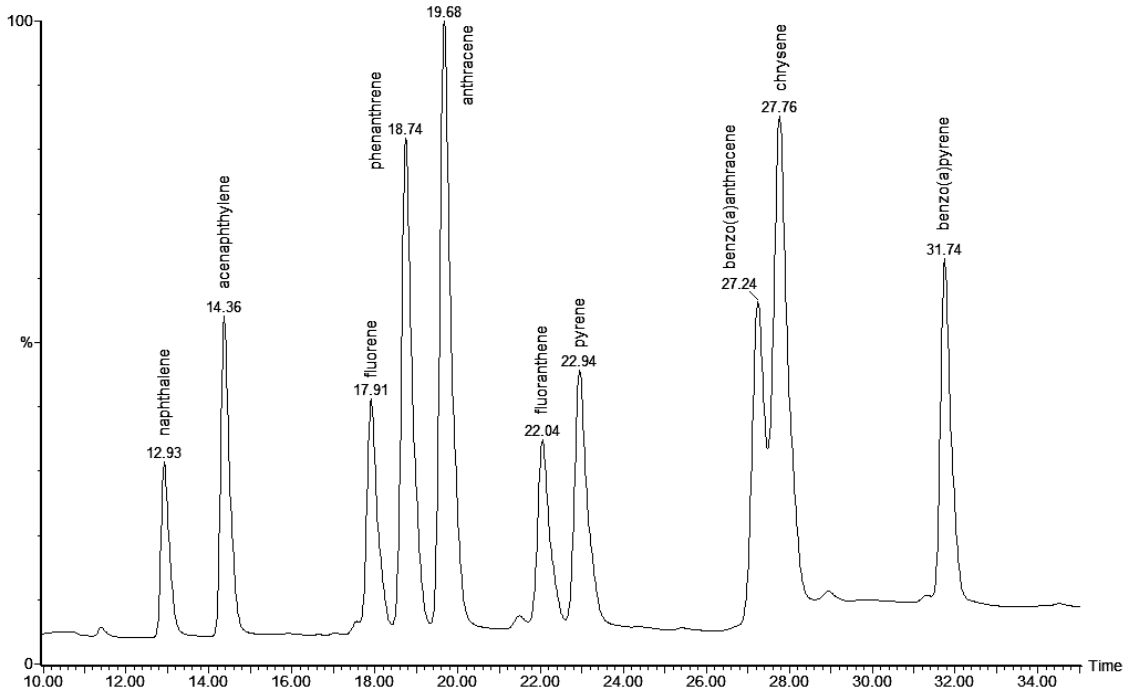

Figure 4.4 Retention times of the parent PAH compounds spiked at 1 mg L^{-1} in colon suspension as analyzed by HPLC followed by diode array detection. Retention time is expressed in minutes.

Limits of detection and recovery of analytes

The limits of detection (LOD) for the PAH hydroxylates, based on a signal to noise ratio of 3:1 was 0.26 to 6.9 µg L^{-1} when determined in MilliQ® water and 0.6 to 31.3 µg L^{-1} for most hydroxylated PAHs when determined in colon suspension (Table 4.1). The LODs for 45OHBaP and 78OHBaP in colon suspension were however much higher compared to the other analytes, reflecting residual interference from background signals (Table 4.1). In general, the LODs are higher than those reported for 1OHP, 4 ng L^{-1}, and 3OH BaP, 51 ng L^{-1}, in urine samples (Hollender et al., 2001). Also for urine samples, Chetiyanukornkul et al. (2002) reported a LOD of 0.1 µg L^{-1} for 1OHP, compared to the value of 0.6 µg L^{-1} observed for the colon suspension in the current investigation. However, the colon suspension can be considered as a more complex matrix with more hydrophobic microbial metabolites, compared to the hydrophilic properties of urinary metabolites. Galceran and Moyano (1996), who also used an LC-MS method for PAH hydroxylate analysis, arrived at higher LOD values of 500 µg L^{-1}. Considering the LOD from these related studies and the matrix complexity from this study, the presented LC-ESI-MS method offers comparable or improved detection of the PAH hydroxylates in colon suspensions.

Table 4.1 Limits of detection (µg L^{-1}) for the PAH hydroxylates. Injection volume was 10 µl for each standard.

	LOD (water) µg L^{-1}	LOD (Colon) µg L^{-1}
1-hydroxy naphthalene	3.5	1.9
2-phenylphenol	1.4	6.0
9-hydroxy fluorene	7.0	17.9
9-hydroxy phenanthrene	1.0	2.1
1-hydroxy pyrene	0.2	0.6
7-hydroxy benzo(a)pyrene	1.2	4.0
4,5-dihydroxy benzo(a)pyrene	3.6	31.3
7,8-dihydroxy benzo(a)pyrene	2.7	21.5

The recovery of the PAH hydroxylates was determined in MilliQ® water (clean matrix) at 0.5 mg L^{-1} and in centrifuged colon suspension (complex matrix) at two concentrations, 0.5 and 0.05 mg L^{-1}. Table 4.2 shows that the recoveries from the MQ water matrix were quite good with values between 82 and 94%. These values are comparable to the recovery values of 80-91% for 1OHP from human urine samples (Li et al., 2002; Ferrari et al., 2003). The relative standard deviation (RSD) values for within-day precision studies (n=3) ranged from 1 to 8%. Only 7OHBaP had a lower recovery percentage of 58%, but the low standard deviation of 4.2% indicates that the results were reproducible. The lower recovery suggests that the solid phase extraction method with methanol as eluting solvent is less selective for the more hydrophobic hydroxylates. The recovery of the pair of dihydroxylates of benzo(a)pyrene gave a high recovery of 121%, reflecting uncertainties associated with calibration and recovery of these two analytes.

For the colon matrix containing 0.5 mg L^{-1} PAH hydroxylates, recoveries ranging from 55 to 89% were obtained. Again, the two dihydroxylates of benzo(a)pyrene were calculated as a non-resolved pair, leading to a recovery of 107%. At lower concentrations of 0.05 mg L^{-1}, the PAH hydroxylate recoveries ranged between 56 and 83% with one lower recovery of 45% for 9OHF (Table 4.2). The lower recoveries of the monohydroxylates, especially at lower concentrations, may be explained by the complexity of the colon matrix itself, which consists of a wide variety of hydrophobic compounds that are not fully removed during sample clean-up. These components easily bind to the packing material of the C18 columns and thus compete with the PAH hydroxylate or parent compounds investigated. Both at concentrations of 0.5 and 0.05

mg L^{-1}, the more hydrophilic PAH hydroxylates – 1OHN, 2PP and 9OHF – appear to have lower recoveries than the more hydrophobic compounds (Table 4.2).

Table 4.2 Recovery (%) and RSD values (%) of PAH hydroxylates fortified in MQ water and SHIME matrix (fortification concentration in parenthesis). (n=3)

	MQ water (0.5 mg L^{-1})	RSD	SHIME matrix (0.5 mg L^{-1})	RSD	SHIME matrix (0.05 mg L^{-1})	RSD
1-hydroxy naphthalene	94 ± 5.6	6	63 ± 3.1	5	67 ± 3.1	5
2-phenylphenol	91 ± 7.0	8	61 ± 3.2	5	56 ± 8.0	14
9-hydroxy fluorene	87 ± 6.9	8	55 ± 6.1	11	45 ± 7.6	17
9-hydroxy phenanthrene	82 ± 6.2	7	89 ± 6.1	7	83 ± 9.3	11
1-hydroxy pyrene	94 ± 5.6	6	72 ± 4.3	6	57 ± 5.8	10
7-hydroxy benzo(a)pyrene	58 ± 4.2	7	87 ± 12.2	14	74 ± 6.8	9
4,5- and 7,8-dihydroxy benzo(a)pyrene	121 ± 1.8	1	107 ± 13.4	12	83 ± 7	8

It is therefore possible that some loss of the more hydrophylic compounds occurred when the extraction column was rinsed with MQ water after sample loading. Likewise, the loading capacity may have been compromised by the complex nature of the colon suspensions. Further refinement of the procedure may thus require the use of less hydrophobic column material such as C8 packing material. Despite the general low recoveries, however, the reproducibility was acceptable at 0.5 mg L^{-1}. The within-day precision RSD value (n=3) ranged from 5 to 11%, showing moderate reproducibility. For example, the within-day precision (n=3) showed a good reproducibility with RSD values between 5 and 11% values for 1OHN, 2PP and 9OHF. At a concentration of 0.05 mg L^{-1}, the reproducibility was good with RSD values between 5 and 11%. For 2PP and 9OHF, the reproducibility was lower with RSD values of 14% and 17% at 0.05 mg L^{-1}. These numbers indicate the usefulness of the presented method to quantify PAH hydroxylates in the intestinal suspension.

The corresponding recoveries for the parent PAHs are given in Table 4.3. For 0.75 mg L^{-1} fortified MQ water, the recoveries were between 114 and 55% with RSD values ranging from 1 to 13.9%. For the more complex SHIME matrix, as expected the recovery was generally lower than those measured for the relatively clean MQ water. Recovery values (obtained from two fortification levels) ranged from 39 to 60% were obtained for the least hydrophobic PAHs, whereas values of 19 to 42% were obtained for the most hydrophobic PAHs, fluoranthene, pyrene, benzo(a)anthracene, chrysene and benzo(a)pyrene. The RSD values (1.1 to 15.2%) (n=3) indicate good to moderate

reproducibility. Besides quantifying PAH hydroxylates in the intestinal suspension, the presented method can thus also be applied for quantification of their respective parent PAHs.

Table 4.3 Recovery (%) and RSD values (%) of PAHs fortified in MQ water and SHIME matrix (fortification concentration in parenthesis) (n=3)

	MQ water (0.75 mg l^{-1})	RSD	SHIME matrix (1.0 mg l^{-1})	RSD	SHIME matrix (0.10 mg l^{-1})	RSD
naphthalene	108 ± 2.1	3	47 ± 3.6	7.7	49 ± 1.7	4.0
acenaphthylene	94 ± 3.0	3.2	60 ± 3.8	6.3	50 ± 2.7	5.1
fluorene	87 ± 1.0	1.2	43 ± 1.1	2.5	51 ± 1.5	3.3
phenanthrene	91 ± 2.3	2.5	42 ± 0.6	1.3	49 ± 1.2	2.4
anthracene	79 ± 9.3	11.7	39 ± 0.4	1.1	46 ± 2.5	6.0
fluoranthene	114 ± 2.5	2.2	41 ± 1.0	2.4	42 ± 3.6	9.3
pyrene	107 ± 1.1	1.0	40 ± 5.1	1.3	38 ± 2.5	7.1
benzo(a)anthracene	55 ± 7.6	13.9	19 ± 2.1	11.3	23 ± 3.5	15.2
chrysene	78 ± 7.3	9.3	26 ± 0.1	4.4	30 ± 1.1	4.5
benzo(a)pyrene	70 ± 9.3	13.3	31 ± 1.9	6.0	40 ± 2.9	7.0

Analysis of hydroxylated PAHs in intestinal suspensions

The proposed method was applied to analyze colon suspension in which PAHs at a 5 mg L^{-1} concentration had been incubated for 24 h at 37°C. This suspension contained a complex microbial community that was comparable to in vivo colon conditions (Molly et al., 1993). No hydroxylated PAHs were recovered from centrifuged colon suspension from which the majority of microorganisms was removed. This showed that no extracellular enzymes were involved in the formation of hydroxylated PAHs. As indicated in Figure 4.3, no hydroxylated PAHs were measured in undosed colon samples which served as a negative control. PAHs that had been incubated in a stomach and small intestinal digestion did not lead to a detection of hydroxylated PAHs either.

In contrast to the negative controls, 1 of the 8 target PAH hydroxylates was detected after 24 h of incubation in colon suspension, namely 1OHP at a concentration of 2.5 µg L^{-1} (Figure 4.5). A small peak was observed for 9OHF, however below the limit of quantification. After incubation of a 1 m aliquot of the sample in glucuronidase and aryl sulfatase, a concentration of 4.4 µg L^{-1} was obtained for 1OHP, suggesting that conjugated metabolites had also been formed. Some of the other PAH hydroxylates investigated were also found but at trace level. 7OHBaP was found at a

1.9 µg L^{-1} concentration, whereas shoulder peaks corresponding to the retention time and m/z ratios for 1OHN and 9OHF were observed but not quantified as they were below the detection limit.

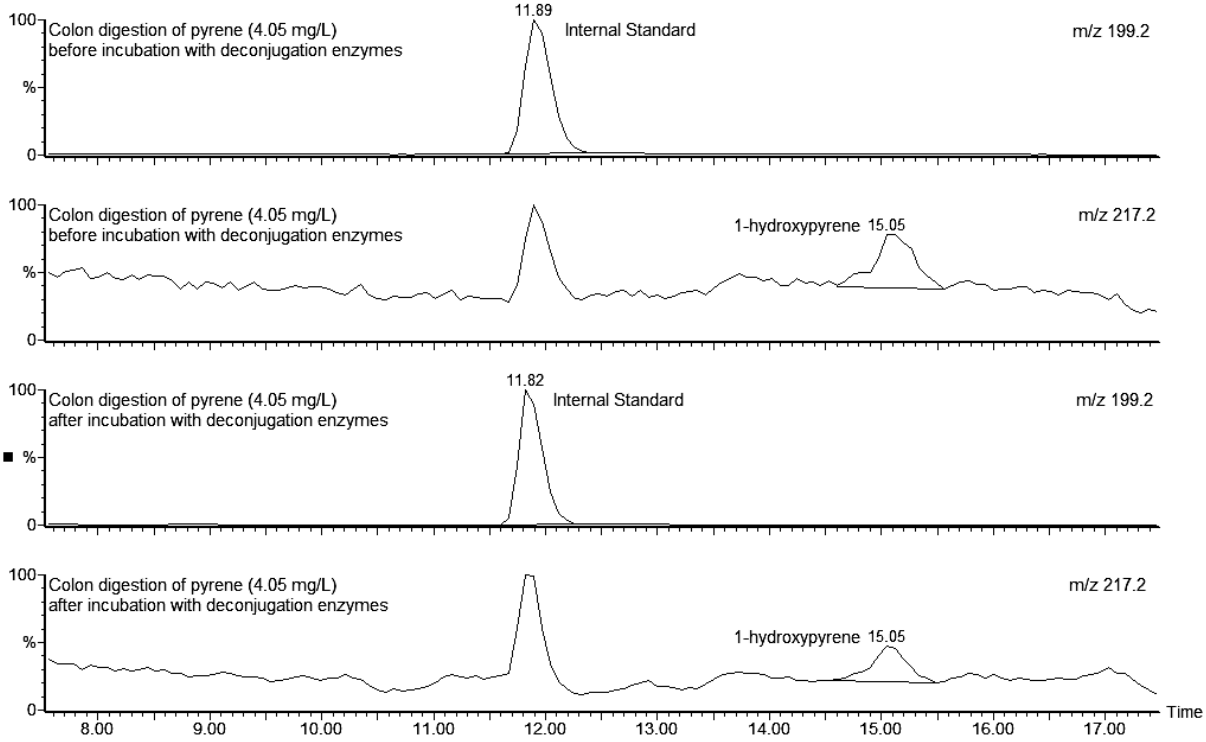

Figure 4.5 Chromatogram for internal standards and 1-hydroxypyrene in colon digests of pyrene at a concentration of 4.05 mg L^{-1}: before (upper 2 chromatograms) and after (lower 2 chromatograms) incubation with β-glucuronidase and arylsulfatase as deconjugation enzymes. The m/z 217.2 peak at retention times of the internal standard probably is a water adduct $M_{I.S.}$+18 ; (199.2+18=217.2)

Conclusion

In general, the LC-ESI-MS procedure provided relatively low detection limits for PAH hydroxylates in colon suspensions. Low recoveries were observed for some analytes with overall good precision for the analytes investigated. The practical application of the method for study of complex colon suspensions was demonstrated to reveal that PAHs may be transformed by colon microbiota to hydroxyl derivatives.

Acknowledgements

Partial funding was provided by the Panel of Energy and Research Development (PERD), Environment Canada. The authors wish to thank Charlotte Boeckaert for preparing the incubation mixtures. We also express our gratitude to Wim De Windt, Joris Roels, Hendrik Nollet and Sofie Dobbelaere for critically reading the manuscript.

Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites

Abstract

Ingestion is an important exposure route for polycyclic aromatic hydrocarbons (PAH) to enter the human body. Although the formation of hazardous PAH metabolites by human biotransformation enzymes is well documented, nothing is known about the PAH transformation potency of human intestinal microbiota. Using a gastrointestinal simulator, we show that human intestinal microbiota can also bioactivate PAHs, more in particular to estrogenic metabolites. PAH compounds are not estrogenic and indeed, stomach and small intestine digestions of 62.5 nmol naphthalene, phenanthrene, pyrene and benzo(a)pyrene showed no estrogenic effects in the human estrogen receptor bioassay. In contrast, colon digests of these PAH compounds displayed estrogenicity, equivalent to 0.31, 2.14, 2.70 and 1.48 nmol 17α-ethynylestradiol (EE2), respectively. Inactivating the colon microbiota eliminated these estrogenic effects. Liquid chromatography – mass spectrometry (LC-MS) analysis confirmed the microbial PAH transformation by the detection of PAH metabolites 1-hydroxypyrene and 7-hydroxybenzo(a)pyrene in colon digests of pyrene and benzo(a)pyrene. Furthermore, we show that colon digests of a PAH contaminated soil displayed estrogenic activity, equivalent to 0.58 nmol EE2, whereas stomach or small intestine digests did not. Although the matrix in which PAHs are ingested, may lower the exposure concentrations in the gut, our results imply that the PAH bioactivation potency of colon microbiota is not eliminated by the presence of soil. Moreover, since PAH toxicity is also linked to their estrogenicity, the PAH bioactivation potency of colon microbiota suggests that current risk assessment may underestimate the risk from ingested PAHs.

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Introduction

Polycyclic aromatic hydrocarbons (PAH) are high priority environmental contaminants because of their toxic, carcinogenic and putative estrogenic or anti-estrogenic properties in the human body. Human exposure to high molecular weight PAHs mainly occurs through oral uptake of charcoil-broiled, grilled and smoked meats (van Maanen et al., 1994) and through ingestion of soil or poorly cleaned vegetables, resulting in exposed doses about an order of magnitude higher than exposure by inhalation (Heisterkamp and van Veen, 1997). The hazardous effects of ingested PAHs come from this PAH fraction that releases from the nutrition, soil or associated organic matter in the intestinal lumen and which, upon intestinal absorption, reaches the intestine enterocytes and liver hepatocytes. In these cells, PAHs may act as ligands to the human aryl hydrocarbon (Ah) receptor, which plays a central role in the toxic response of specific aromatic hydrocarbons by the regulation of typical human biotransformation enzymes (reviewed by Hankinson, 1995).

The risk from orally ingested PAHs is currently thought to be reduced when co-ingested soil or fibres decrease the intestinal PAH absorption and hence, bioavailability (De Kok and van Maanen, 1992). The majority of ingested PAHs would pass harmlessly through the gastrointestinal (GI) tract without being transformed by human enzymes to hazardous metabolites. However, this assumes that no microbial biotransformation of PAHs occurs. The human GI tract harbors an incredibly diverse microbial community, which typically performs fermentative processes, but which is also capable of transforming xenobiotic compounds (Macdonald et al., 1983; Ilett et al., 1990; Aura et al., 2002). Hence, if microbial PAH biotransformation in the human colon is possible, the susceptibility of the colon epithelium to bioactive PAH metabolites may increase the health risks that are associated with non-absorbed PAHs that reach the colon. To date, no information is available on the PAH bioactivation potency from human colon microbiota. To evaluate this, we looked in this study at PAH estrogenicity, since several PAH metabolites structurally resemble steroidal hormones that bind the human estrogen receptor (Ariese et al., 2001), which could thus lead to estrogenic or anti-estrogenic activity *in vivo.*

We opted for an in vitro approach to specifically look for microbial biotransformations and thus avoid possible interference from colon epithelium enzymes that would be present in an in vivo approach. Pure PAH compounds and a PAH contaminated urban soil were incubated in the stomach, small intestine and colon suspensions from a simulator of the human GI tract. Given the aromaticity of PAHs, we

used a modified aryl hydrocarbon (Ah) receptor yeast assay (Miller, 1997) to investigate whether the PAHs in the different digests could activate the human Ah receptor and subsequently induce signal transduction. Besides this, we investigated the estrogenicity of the PAH incubated digests by monitoring activation of the human estrogen receptor in a modified estrogen receptor yeast assay (Routledge and Sumpter, 1996). In addition, we applied a newly optimized liquid chromatography – mass spectrometry (LC-MS) protocol to detect whether PAH metabolites were formed during incubation.

Materials and Methods

Chemicals

PAH parent compounds napthalene, phenanthrene, pyrene and benzo(a)pyrene were reagent grade and obtained from Sigma-Aldrich (Bornem, Belgium). To avoid solubility problems in the incubation tests, PAHs were first dissolved in ethanol prior to digestion. All stock solutions were prepared in amber glass bottles and stored in the dark at 4 °C. Hydroxy-PAH metabolites 1-OH naphthalene, 9-OH phenanthrene, 1-OH pyrene and 7-OH benzo(a)pyrene were reagent grade and also obtained from Sigma-Aldrich.

Incubations

Incubations of PAHs and soils were performed in batch by sampling gastrointestinal suspension from the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This dynamic model of the gastrointestinal tract consists of 5 compartments representing the stomach, small intestine, colon ascendens, transversum and descendens, respectively (Figure 5.1). The colon suspension contains *in vitro* cultured microbiota which were isolated from human feces and which are representative for the *in vivo* colon microbial ecology after a growth stabilization period in the different colon compartments (Molly et al. 1993). A typical stomach digestion consists of an incubation of PAHs or PAH contaminated soil samples for 3 hours at pH 1.5 at 37°C. A small intestine digestion consists of an incubation for 5 hours at pH 7 at 37°C in the presence of bile salts (0.2 mmol L^{-1}) and pancreatic enzymes supplemented as pancreatic powder from porcine origin (0.4 g L^{-1}). A colon digestion consists of an incubation with colon microbiota for 48 h at 37°C, withdrawn from the colon vessels of the SHIME reactor. Some samples were incubated with

inactive colon microbiota. For this, colon microbiota were autoclaved for 30 minutes (121°C, 1 bar overpressure). Incubation of PAH standard compounds in stomach, small intestine and colon digests occurred at a concentration of 20 µmol L^{-1}. This concentration is normally not encountered in the gastrointestinal tract, but gave us more possibilities to study microbial PAH metabolism in depth. Gastrointestinal digestion experiments on soil samples were done as previously described (Van de Wiele et al., 2004) to simulate a hypothetical soil ingestion of 5 g d^{-1} by children (stomach 50mL, small intestine 60mL and colon 100mL). To avoid photocatalytic effects, all digestions were performed in amber flasks. After the respective incubations, samples were centrifuged at 3000×*g* for a duration of 10 minutes to remove most of the particulates and biomass. The supernatants were subsequently stored at -20°C prior to analysis.

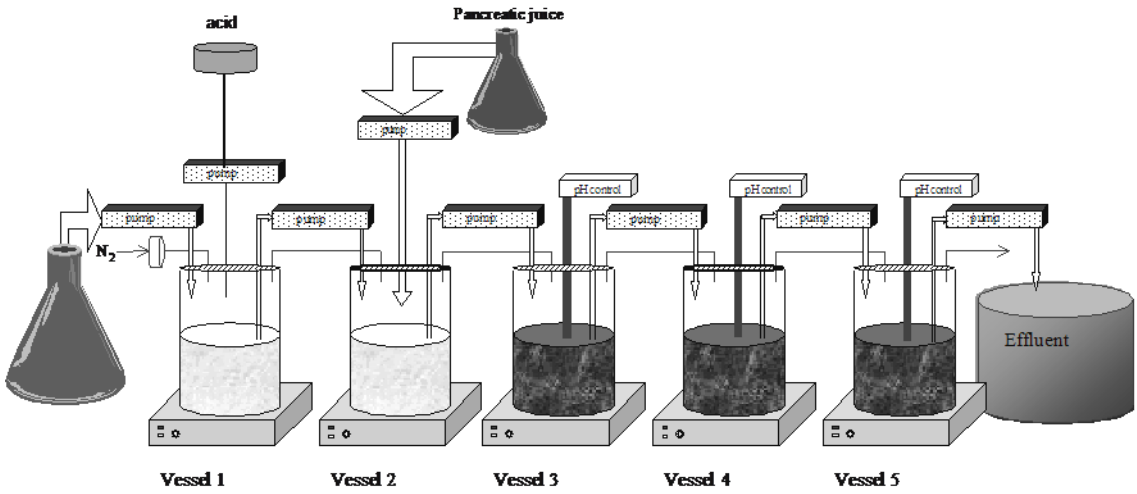

Figure 5.1 Schematic representation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Vessels 1 to 5 respectively simulate conditions from the stomach, small intestine, colon ascendens, colon transversum and colon descendens.

Sample treatment

PAH parent components and PAH metabolites were extracted from the digests by performing a liquid/liquid extraction in which the digest and ethylacetate were mixed in a 1:1 ratio. The ethylacetate fraction was subsequently put in a rotavapor to remove most of the solvent. The remainder of the solvent was removed under a gentle stream of nitrogen gas and finally replaced by dimethylsulfoxide which is a suitable solvent to use in bioassay tests. For chemical analysis of the samples using LC-MS, sample

aliquots were subjected to a solid phase extraction using PrepSepTM C18 (250 mg) (Fisher Scientific, Edmonton, Canada). PAH hydroxylates were eluted with methanol.

PAH conjugate analysis

To check whether conjugated PAH metabolites were formed in the different digests, samples were also incubated in the presence of β-glucuronidase and aryl sulfatase, both obtained from Sigma–Adrich (Belgium). After the PAH parent compounds had been incubated in SHIME suspension, a 1 mL aliquot of these samples were diluted in 1mL 0.1 M acetate buffer and the pH was adjusted to 5 with sodium hydroxide. A volume of 400 µl β-glucuronidase (100U mL^{-1}) and 250 µl aryl sulfatase (60U mL^{-1}) were added and the mixture was incubated for 6 h at 37 °C to hydrolyze the PAH conjugates.

Bioassays

For the bioassays, we used a modified protocol from De Boever et al. (2001) that was based on the protocol developed by Routledge and Sumpter (1996) for the yeast estrogen bioassay and Miller (1997) for the yeast Ah bioassay. Details on the yeast Ah bioassay, the yeast estrogen bioassay and the preparation of the medium compounds have been described before (Routledge and Sumpter, 1996; Miller, 1997). In brief, these researchers transformed *Saccharomyces cerevisiae* with the human Ah receptor gene and the human estrogen receptor (ERα) gene, together with expression plasmids containing responsive elements and the *lacZ* reporter gene (encoding the enzyme β-galactosidase). The expression of β-galactosidase is triggered by test chemicals, which upon binding to the respective receptors induce the conformational change necessary for binding of the receptor/ligand dimer to the responsive elements. This β-galactosidase activity is quantified at 540 nm by the conversion of the chromogenic substance chlorophenol red-β-D-galactopyranoside into chlorophenol red. The bioassay response is expressed as the absorbance at 540 nm divided by the optical density at 630 nm $(A540/A\ 630)_{net}$. Positive signals in the Ah receptor assay were typically expressed as percentage equivalence to 200 nM benzo(a)pyrene which arbitrarily corresponded to a bioassay response of 100 %. Similarly, estrogenic activity of the samples was expressed as percentage equivalence to 6.96 nM 17α-ethynyl estradiol which elicited a 100 % response in the estrogen receptor bioassay (De Boever et al., 2001). To make sure that background signals from gastrointestinal suspensions of soil or food matrices did not interfere with the detection of estrogenic signals in the bioassays, corrections were made in a set of negative control

experiments by subtracting the response of a PAH containing digest with that from a blank digest without PAHs (see supplemental material). The bioassays were performed in 96-well plates in which 10 µL of the test compounds were tested and incubated with 240 µL of the genetically modified yeast (optical density of 0.25 at 610nm). Serial dilutions of the test compounds were made in dimethylsulfoxide which allowed to generate dose-response curves for doses (ordinate) versus activity (abscissa). The data were fitted by a 4 parametric logistic model using the Marquardt-Levenberg algorithm (Sigmaplot 4.0, SPSS Inc., Chicago, Illinois, USA) (De Boever et al., 2001).

Negative controls

Since we specifically examined the effect of gastrointestinal digestion processes on the production of estrogenic PAH metabolites, we corrected for background signals coming from the digestion matrix as such. Therefore we tested blank digest suspensions, which are typically the stomach, small intestinal or colon suspension from the SHIME reactor to which no PAHs had been added. No positive responses were expected from the blank stomach or small intestinal digests, but the colon suspension from the SHIME reactor is a complex mixture of microbiota and hundreds of different metabolites among which some compounds may posses pseudo-estrogenic properties. The latter may be capable of binding the human estrogen receptor in the bioassay test and hence lead to a small, yet positive response. Blank stomach and small intestine digests indeed did not induce a significant response in the yeast estrogen bioassay (Figure 5.2a), hence no corrections for these digests were performed in later experiments. We noted however an inhibition in yeast growth when the bioassay was performed on blank small intestine digests. This inhibtion was previously explained by the presence of bile salts (De Boever et al., 2001). These compounds are present in the small intestine digests at 0.2 mmol/L and may disrupt the membrane integrity of the yeast cell wall. In contrast to the stomach and small intestine digests, the blank colon digest elicited a positive response of 28% EE2 equivalence at the highest concentration (Figure 5.2a), thus making corrections on experimental data necessary. This value may seem high, but mainly originated from low absorbance values at 630 nm, which increases the final response, calculated as $(A540/A630)_{net}$. All dose-response curves of colon digests were hence corrected for this matrix background signal.

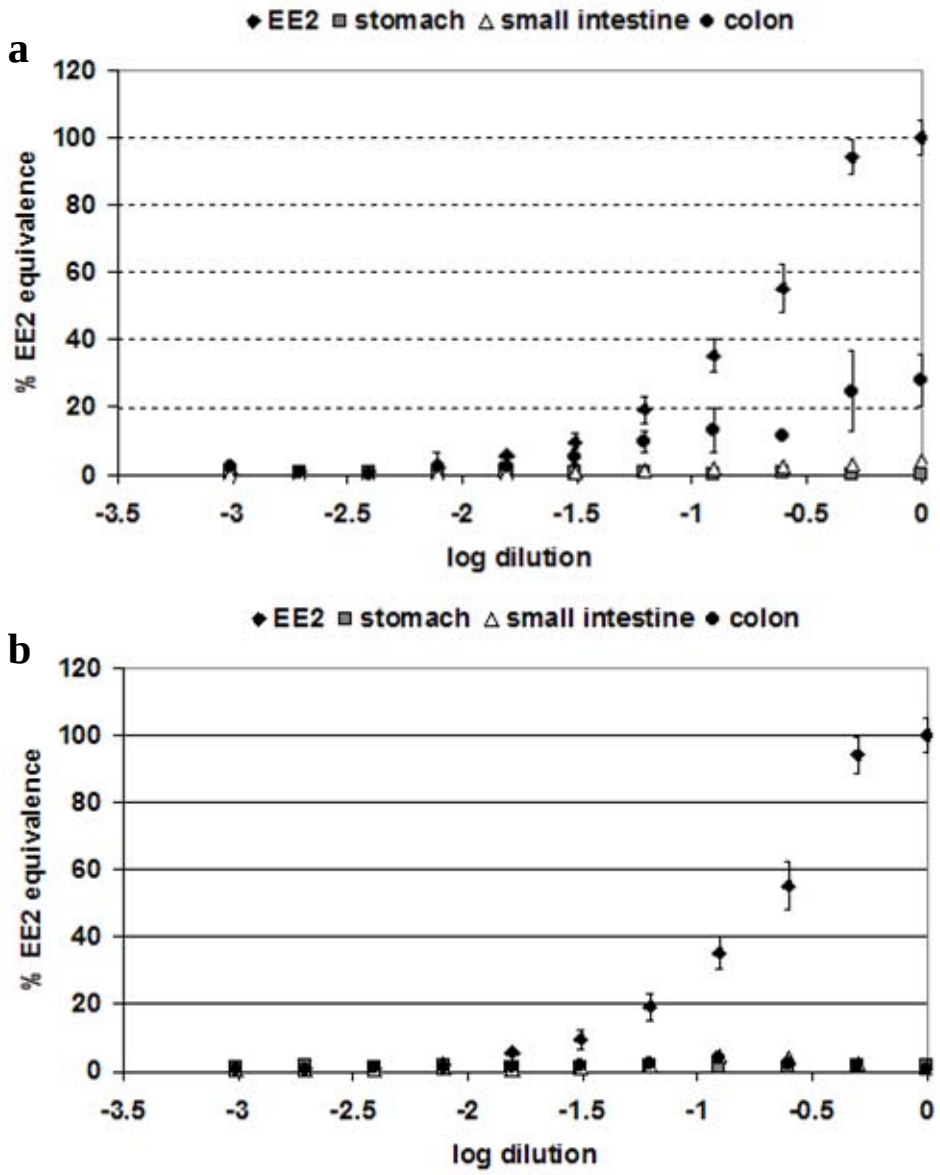

Figure 5.2 Dose-response curves in the estrogen yeast assay for negative control samples. Values are averages of 4 replicates. Error bars represent standard deviation and may disappear in the datapoint if too small.

a. blank stomach, small intestine and colon digests to which no PAHs had been dosed, were screened for estrogenic effects in the bioassay.

b. stomach, small intestine and colon digests on a clover soil, rich in phytoestrogens were tested for their estrogenic response in the yeast estrogen bioassay. The estrogen response for the colon digest has been corrected for the matrix background response coming from colon suspension (Figure 5.2a).

PAH contaminated soil samples were also included in this research and they too were subjected to the digests from the SHIME model. Besides PAHs and their possible metabolites, these soils may also contain pseudo-estrogenic compounds, phytoestrogens for instance. We examined whether the presence of phytoestrogens in

a soil sample could be of any concern in the yeast estrogen bioassay. We therefore incubated a clover (Family Leguminosae) soil, putatively rich in phytoestrogens to a stomach, duodenal and colon digest and analyzed these samples in the bioassay. Very low estrogenic signals of 5.6% EE2 equivalence were observed for stomach or duodenal digests on this soil (Figure 5.2b). A colon digestion on this leguminosae soil did lead to a slightly positive response in the lower 21 to 24 dilution range, however not significantly different from the blank colon suspension. After correction for this colon background signal, a maximal estrogen response of 4.5% was obtained (Figure 5.2b). We therefore concluded that no corrections for possible phytoestrogens in the PAH contaminated soil were needed, since on the latter soil there was primarily growth of Poaceae, which produce phytoestrogens at much lower concentrations than Leguminosae (Grippo et al., 1999).

PAH analysis (GC-MS)

Sample treatment for and determination of PAHs were performed by the Environmental Research Centre (Erembodegem, Belgium). Briefly, PAHs from pellets were extracted by a 1:1 aceton/hexane mixture using an ASE® 200 (Accelerated Solvent Extractor, Dionex, Sunnyvale, CA, USA). PAHs from supernatants were extracted with dichloromethane. Analysis of the PAH content in the extracts was performed according to a standardized method (Method EPA8270, Environmental Protection Agency, USA) by gas chromatography coupled with mass spectrometry (GC–MS). The MS detector used was a quadrupole mass spectrometer (Trace-MS, Fisons/Thermoquest, Belgium). The detection limit for the different PAH components was 0.2 µg L^{-1}. The quantification limit was 0.4 µg L^{-1}. The extraction efficiency of the sample preparation step prior to PAH analysis was between 80 and 110%, as determined with the reference soil CRM535.

Hydroxy-PAH analysis (LC-MS)

LC-MS analysis of the samples for hydroxy-PAHs was performed according to Van de Wiele et al. (2004b). The identity of hydroxy-PAH metabolites in the samples was confirmed by using synthetic standards of these metabolites and compare the HPLC profiles from the colon digests with those from the standards. Briefly, all samples for LC-MS analysis were subjected to solid phase extraction using PrepSep™ C18 columns (250 mg) (Fisher Scientific, Edmonton, Canada). Sample volumes of 5ml were loaded on the columns and washed with 10 mL of Milli-Q ® water and

subsequently, the target analytes were eluted with 10 mL of methanol. One mL aliquots were subsampled and stored in amber vials at 4°C prior to LC-MS analysis. HPLC analysis was performed using a Waters 2695 (Milford, MA, USA) separation module. The selected column was a 2.1 mm×100 mm, 3.5 µm particle size, Waters XTerra MS C18 column (Milford, MA, USA) which was kept at a constant temperature of 26 °C. The binary eluent system consisted of methanol:water 90:10 v/v (eluent A) and methanol:water 10:90 v/v (eluent B). Mass spectrometry analysis was performed with a Quattro Ultima Mass spectrometer (Micromass Technologies, Manchester, UK) that was equipped with an electrospray interface operating in the negative ion mode. Instrumental control and data acquisition was performed with MassLynx software version 3.5. The electrospray ionization source was operated at 90°C, desolvation temperature 200 °C, cone voltage 61 V, and a capillary voltage of 2.74 kV. Nitrogen gas served as the cone gas (flow rate of 159 L h^{-1}), desolvation gas (490 L h^{-1}) and nebulizer gas (set to maximum). The detector multiplier voltage was set to 650V. Selected ion monitoring was employed for quantitative analysis monitoring the $(M-H)^-$ of m/z for the PAH hydroxylates.

Results and Discussion

Bioactivation of pure PAHs

Due to their moderate to high degree of aromaticity, we expected pure solutions of naphthalene, phenanthrene, pyrene and benzo(a)pyrene to test positive in the Ah bioassay. Naphthalene, 200 nM, displayed 0.4 % benzo(a)pyrene equivalence whereas 200 nM phenanthrene and 200 nM pyrene displayed 15.1 % and 48.2 % benzo(a)pyrene equivalence. PAH compounds are not estrogenic and up to 16 µM of the four pure PAHs indeed did not induce an estrogenic response in the estrogen bioassay. Similarly, separate stomach and small intestine digests of the four PAHs did not show a significant estrogen response (Figure 5.3). In contrast, PAHs from colon digests became estrogenic. Conversion of the % EE2 equivalence values, depicted in Figure 5.3, to equivalent EE2 concentrations resulted for colon digests of 62.5 nM pyrene, into 2.70 nM EE2 equivalence, for phenanthrene, 2.14 nM EE2 equivalence, for benzo(a)pyrene, 1.48 nM EE2 equivalence and for naphthalene 0.31 nM EE2 equivalence. This PAH bioactivation was only evident upon the colon digestion. This shows the selectivity of the colon digestion towards an increase in estrogenicity whereas no increased aryl hydrocarbon response was detected, compared to stomach or small intestine digests. To make sure that the observed effects were not coming

from the matrix background of the colon interacting with PAHs, we incubated PAHs in a heat inactivated colon suspension. The removal of microbial activity markedly reduced the increase in estrogenic activity (Figure 5.3). This finding indicates that the risk for PAH bioactivation along the gastrointestinal tract is not exclusively associated with human biotransformation enzymes from the enterocytes in the small intestine epithelium and colonocytes in the large intestine epithelium (Autrup et al., 1978; De Kok and van Maanen, 2002; Doherty and Charman, 2002), but that colon microbiota can also bioactivate PAHs.

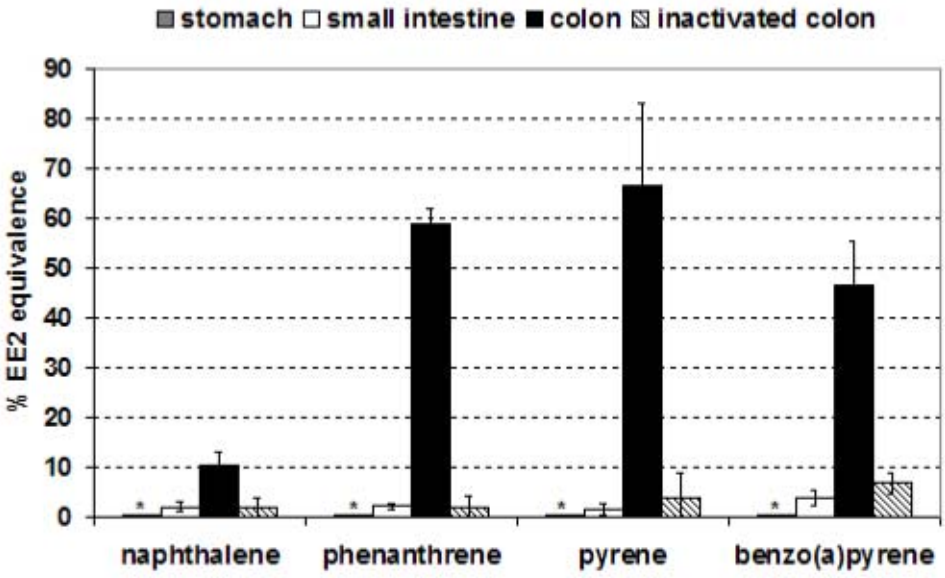

Figure 5.3 Estrogen response of naphthalene, phenanthrene, pyrene and benzo(a)pyrene incubated at 62.5 nmol/L in stomach, small intestine, colon digests and digests with inactivated colon microbiota. Error bars represent standard deviation values of 4 replicates. * none of the stomach digestions gave a significant response in the estrogen bioassay.

Bioactivation of soil-bound PAHs

We then evaluated the significance of this process using lower, more realistic concentrations obtained from a former urban playground soil, contaminated with 49±1.5 mg PAH kg^{-1} soil DW by years of atmospheric deposition. Children form the largest risk group for soil ingestion due to their typical hand-mouth behaviour and small body weight. Hence, we simulated the GI tract of a child, hypothetically ingesting 5 g soil d^{-1}. GC-MS analysis previously showed that the released PAH fraction from the soil matrix was highest in the stomach digest (18±5.3 µg L^{-1}), followed by the small intestine digest (3±1.1 µg L^{-1}) and the colon digest (2±0.3 µg L^{-1}) (Van de Wiele et al.,

2004d). This corresponded to a maximal Ah bioassay response for the stomach digest of 41±2.9 % benzo(a)pyrene equivalence, the small intestine 27±1.4 % and the colon 22±2.6 % (Figure 5.4).

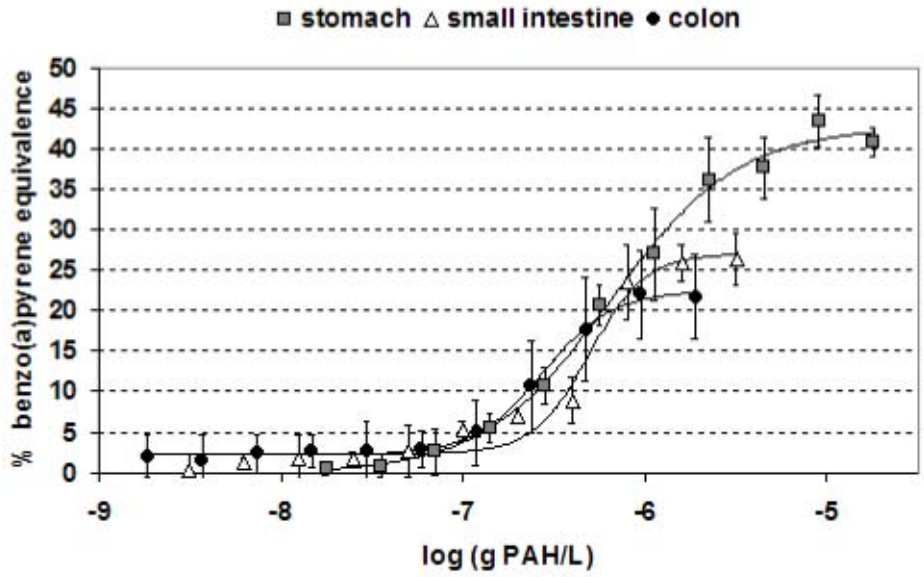

Figure 5.4 Dose-response curve of stomach, small intestine and colon digests of a PAH contaminated playground soil in the Ah receptor yeast bioassay, expressed as percentage benzo(a)pyrene equivalence in function of released PAH concentration in the respective digests. Values are averages of 4 replicates. Error bars represent standard deviation and may disappear in the datapoint if too small.

Based on the role of the human Ah receptor in the toxicity of specific aromatic hydrocarbons, these findings would normally indicate that the colon digest represents the lowest risk for PAH bioactivation. Surprisingly, the trend in estrogenic activity was the inverse of observed PAH release or Ah bioassay response. Similar to the estrogen bioassay results on pure PAHs, there was negligable induction of estrogenic activity upon the stomach and small intestine digestion (Figure 5.5). However, an average value of 20.1±0.84 % EE2 equivalence was observed upon a colon digestion of the contaminated soil (Figure 5.5). We infer that the PAH bioactivation potency from colon microbiota also occurs at lower and relevant concentrations for human exposure and that the presence of soil does not eliminate this potency. To bring these numbers in perspective, it is interesting to note that the observed estrogenicity is quite considerable, in comparison to the intestinal digestion of soy germ powder at relevant adult concentrations as performed by De Boever et al. (2001). De Boever et al. (2001) noted that the presence of soy isoflavones genistein, glycitein and daidzein resulted in

an estrogenic response of 35 ± 13.8% equivalence to 6.96 nM EE2. Taking into account these numbers, it can be concluded that the microbial PAH bioactivation to compounds with estrogenic properties may be physiologically important.

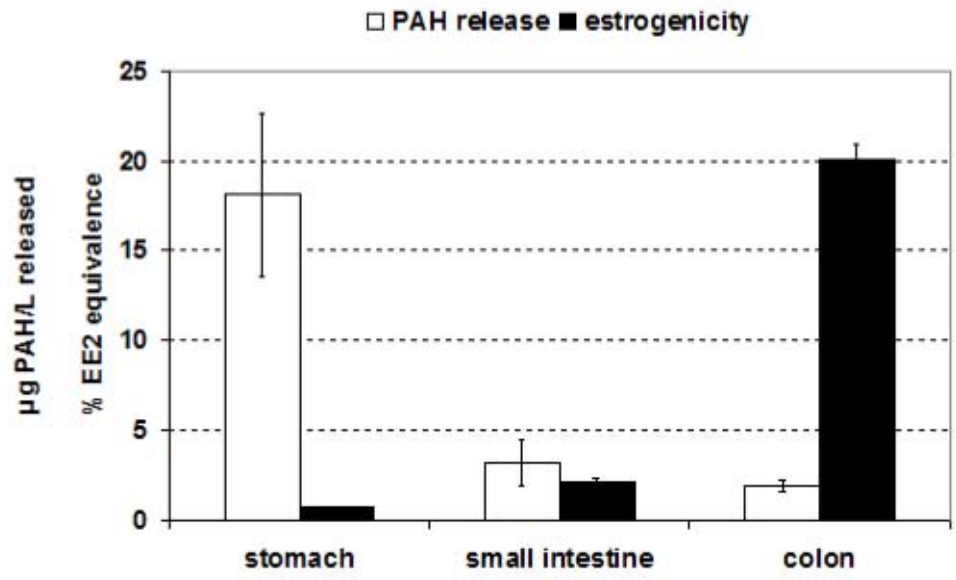

Figure 5.5 Released concentrations of PAHs and estrogen response upon stomach, small intestine and large intestine digests on PAH contaminated soil samples. Error bars represent standard deviation values of 4 replicates.

Soil organic matter or nutritional fibres are known to lower the fraction of a contaminant that can be absorbed by the intestine (van Schooten et al., 1990; O'Neill et al., 1991; Oomen et al., 2000). This would theoretically lower the risk from ingested contaminants since bioactivation by human biotransformation enzymes will be reduced due to a lower bioavailability. To test this hypothesis, we compared the estrogenicity from colon incubated PAHs in the presence and absence of soil by calculating the bioactivation potency of the digests as estrogenicity / aromaticity. We divided – at equimolar concentrations – the % EE2 equivalence of the different digests by their respective % benzo(a)pyrene equivalence. At equimolar concentrations of 8.03 nmol PAH L^{-1}, this ratio was for naphthalene, 0.93, for phenanthrene, 2.16, for pyrene, 0.98 and for benzo(a)pyrene, 0.12, whereas the colon digest of the PAH contaminated soil gave a ratio of 0.88, the same order of magnitude as the ratios for pure PAH compounds and significantly higher than the ratios for the stomach soil digest, 0.016, or small intestine soil digest, 0.077. These findings provide further evidence that the presence of a soil matrix does not eliminate the PAH bioactivation by colon microbiota and that the estrogenic potency of soil derived PAHs does not significantly decrease if compared to pure PAHs.

Analytical detection of PAH metabolites

PAH metabolites that typically have estrogenic properties, are hydroxylated derivatives, due to their structural similarity to natural estrogens (Hirose et al., 2001; Fertuck et al., 2001a; Fertuck et al., 2001b). Hence, in a next step of the research, we screened with LC-MS for the presence of hydroxy-PAHs by analyzing the respective colon digests of 20 µmol L^{-1} pure PAH compounds. The identity of PAH metabolites was confirmed by comparing the HPLC profiles and MS spectra of the colon digests with those from chromatographic synthetic standards of several hydroxy-PAHs. The developed protocol had reasonably low detection limits for 1-hydroxy napthalene, 9-hydroxy phenanthrene, 1-hydroxy pyrene and 7-hydroxy benzo(a)pyrene (Table 4.1) (Van de Wiele et al., 2004b). From all colon digests, only the pyrene digest tested positive for a hydroxylated PAH metabolite with 1-OH pyrene at a concentration of 2.5 µg L^{-1}. Hydroxylated PAHs were not found in the stomach and small intestine digests. Glucuronidated or sulfated PAH conjugates are also typical biotransformation products from eukaryotic organisms (Cajthaml et al., 2002). Since the concentration of fungi and yeasts in the colon suspension amounted to 4.3±0.6 log CFU mL^{-1}, we tested whether PAH conjugates were present in colon digests of pure PAH compounds. Glucuronidase and arylsulfatase typically cleave off glucuronic acid or sulfate groups from conjugated PAHs, regenerating the hydroxylated PAH metabolites (Cajthaml et al., 2002). After incubating the extracts of the colon digests in the presence of glucuronidase (100 U mL^{-1}) and arylsulfatase (60 U mL^{-1}) for 6 hours at 37°C, we found higher concentrations of 1-OH pyrene (4.4 µg L^{-1}) and a new metabolite, 7-OH benzo(a)pyrene (1.9 µg L^{-1}). No hydroxylated PAHs were retrieved from inactivated colon samples. Although other PAH hydroxylates may have formed than those tested during LC-MS analysis, these analytical data show that PAH bioactivation by colon microbiota may result from hydroxylated PAH metabolites.

Biological plausibility and relevance of PAH bioactivation

The formation of hydroxylated PAH metabolites and especially the increased estrogenicity by human colon microbiota bring up two questions. Are the observed transformations plausible for the *in vivo* human gastrointestinal tract and to what extent can bioactive PAH metabolites contribute to the total risk from oral PAH exposure?

To answer the first question, literature shows that resident gut microbiota may influence xenobiotic metabolism from the intestinal epithelium (Hooper et al., 2001). Additionally, microbial glucuronidase activity in the intestine sometimes cleaves off

glucuronic acid groups from excreted human conjugated metabolites, thus regenerating the more bioactive hydroxylated intermediates (Aura et al., 2002). These reports describe indirect effects of intestinal microbiota towards xenobiotic metabolism. However, our findings indicate a direct metabolism effect of human colon microbiota towards PAH parent compounds, since the in vitro approach used in the current study eliminated possible interferences by intestinal epithelium enzymes. The observed biotransformation and bioactivation reactions originate from a microbial community which resembles that of the *in vivo* intestinal lumen both in composition and in metabolic activity. Rather than containing the less active microbiota from fecal matter, the microbial community from the used *in vitro* method is more representative of the different parts of the human colon (Molly et al., 1993).

As suggested by the LC-MS results, the colon microbiota formed hydroxylated PAH metabolites, which may seem uncommon since this oxidative step would occur in an anaerobic environment as shown by redox potential values from the colon suspension that varied between -180 mV and -230 mV. The latter values are well within the range of -145 mV to -250 mV reported for the colon in vivo (Bowler et al., 2001; Chourasia and Jain, 2003). Yet, oxidative reactions by intestinal bacteria from humans, mice and rats have been described for the conversion of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) to its reportedly mutagenic 7-keto derivative (7-OHIQ) (Rumney et al., 1993). Additionally, *Enterococcus faecalis* even performs aromatic hydroxylation reactions in the intestine *in vivo* (Huycke and Moore, 2002). It is therefore not unlikely that intestinal microbiota may hydroxylate PAHs, also given the fact that anaerobic PAH hydroxylation has been reported by microorganisms, beit in sediments (Karthikeyan and Bhandari, 2001). These studies on oxidative reactions by intestinal microbiota and anaerobic PAH biotransformations may thus support our findings, which need further study to identify which microorganisms bioactivate PAHs.

To answer the second question concerning the contribution of the observed effects to the total risk from PAH ingestion, further research is warranted. Yet, the microbial PAH bioactivation to estrogenic metabolites may constitute an increased health risk when the human body is orally exposed to contaminated soils. The human colon epithelium is 20% more permeable to 17α-estradiol than the human small intestine epithelium (van der Bijl and van Eyck, 2003) and also has a higher permeability to hydrophobic compounds in general (Ungell et al., 1998). PAH metabolites with structures resembling steroidal hormones may thus exhibit weak estrogenic or antiestrogenic activity *in vivo* (Ariese et al., 2001). Since PAHs that reach the colon will be biotransformed by colon microbiota, we conclude that, in the *in vivo* situation, the

colonic epithelium – which has estrogen receptors – may be subjected to hazardous effects from microbial PAH metabolites. The equivalent EE2 response of 20 % for the colon incubated environmental sample (Figure 5.5) indicates that the observed activation of the human estrogen receptor is significant. Still, it must be kept in mind that a positive response in ER-reporter gene assays such as that from the present study, does not necessarily predict endogenous transcription (Gozgit et al., 2004). Gozgit et al. (2004) noted that several PAHs induced activity in ER-reporter gene assays but that these PAHs did not upregulate estrogen-responsive genes. The authors conclude that the ER-reporter gene assays may detect concentrations of toxicants that are not physiologically active. In light of these recent findings, the estrogenic response from microbial PAH bioactivation in this study needs careful interpretation. However, the finding of 1-OH pyrene and 7-OH benzo(a)pyrene as metabolites from human colon microbiota is something that is not anticipated from current scientific knowledge or risk assessment studies. Comparison of our findings from active to those from inactivated colon microbiota shows us that the microbial bioactivation potency is a factor 12 higher than would be currently expected in risk calculations. Moreover, the time during which bioactive hydroxy-PAHs could react with colonocytes is also considerably longer (up to 72 hours) than the residence time in human enterocytes or hepatocytes (8 hours). Additionally, if taken up by colonocytes, the hydroxy-PAHs are typical metabolites that are more easily metabolized by human biotransformation enzymes to for example potent carcinogens such as b(a)p-r7,t8-dihydrodiol-t9,10-epoxide (Kim et al., 1998). Clearly, these literature reports on human PAH metabolism and our findings of PAH bioactivation by colon microbiota indicate the importance of conducting future work in which the relative importance of the human bioactivation processes versus the microbial bioactivation processes should be compared.

Conclusion

In summary, our results reveal that human colon microbiota can directly bioactivate PAHs, a potency that has not been reported before. As indicated by the analysis of a PAH contaminated environmental sample, we also show that the presence of soil does not eliminate this microbial bioactivation potency. We therefore conclude that risk calculations that are solely based on human biotransformation enzymes, may underestimate the risk from ingested aromatic contaminants because it does not consider the bioactivation processes described here.

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CHAPTER 6

Prebiotic chicory inulin exerts chemopreventive activity towards bioactivation of polycyclic aromatic hydrocarbons by intestinal microbiota

Abstract

Inulin is frequently studied for its prebiotic activity as it stimulates health-promoting bacteria in the human colon. Inulin is also hypothesized to exert chemopreventive effects towards hazardous biotransformation reactions from the resident colon microbiota towards potential toxicants in the gut lumen. Using a Simulator of the Human Intestinal Microbial Ecosystem (SHIME), we investigated whether inulin addition inhibited the conversion of polycyclic aromatic hydrocarbons (PAH) to estrogenic metabolites by human colon microbiota. Additionally, prebiotic effects were evaluated by monitoring the microbial community from the colon compartments in terms of metabolic activity and community structure. Yeast estrogen bioassay data revealed that inulin addition significantly decreased the estrogenicity of PAH incubated samples from the ascending (39% decrease) and transverse (14% decrease) colon compartment, whereas inulin did not exert chemopreventive effects in the descending colon. Interestingly, this colon-specificity showed parallels with the prebiotic effects. Conventional culture-based techniques and PCR-DGGE analysis on the SHIME colon suspension revealed significant bifidogenic effects during inulin treatment in the ascending colon whereas the overall microbial community kept relatively unchanged. Additionally, short chain fatty acid production increased with 44%, 23% and 33% in the ascending, transverse and descending colon while ammonia concentrations significantly decreased in the ascending colon. This indicates that the prebiotic effects from inulin may also purport inhibitory effects towards PAH bioactivaiton. From the real time PCR data, which showed bifidogenic effects (> 1 log CFU mL^{-1}) throughout the entire colon, we infer that specific conditions from the proximal colon region promote the chemopreventive effects from inulin the most.

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Introduction

Human exposure studies reveal that polycyclic aromatic hydrocarbons (PAH) are more likely to be ingested than inhaled by the incidental uptake of contaminated soils or the consumption of processed foods such as charcoal-broiled, grilled and smoked meat (De Kok and van Maanen, 2000). Dietary exposure in particular significantly contributes to total PAH exposure and depending on the individual life style, the total daily PAH-intake may vary between 25 and 300 µg d^{-1} (van Maanen et al., 1994). The oral bioavailability is assumed to be quite low, below 10% (van Schooten et al., 1997), because of complexation effects with the soil matrix or dietary constituents that limit the available fraction for intestinal absorption (Chapter 3; Van de Wiele et al. 2004d). Hence, a substantial part of the ingested PAHs reaches the colon and rectum, thus presenting a putative target for PAH-induced carcinogenicity. In order to exert toxic effects in colon and rectum, PAHs need to be bioactivated (De Kok and van Maanen, 2000). It has been demonstrated that colonic tissue is able to metabolize PAHs similarly as found in other tissues (Autrup et al., 1978), but interestingly, the research results from Chapter 5 show that colon microbiota can also convert PAHs to bioactive components (Van de Wiele et al., 2004c), more specifically metabolites with estrogenic properties.

The bioactivation potency was illustrated previously by several studies that show that colon microbiota are involved in colorectal cancer by the production of carcinogenic metabolites (Hirayama et al., 2000; Hughes and Rowland, 2000; Aura et al., 2002; Gill and Rowland, 2002). As an example, intestinal microbiota from humans, mice or rats were responsible for the conversion of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) to the mutagenic 7-keto derivative (7-OHIQ) (Rumney et al., 1993; Kassie et al., 2001). These reports and the newly discovered PAH bioactivation by colon microbiota from Chapter 5, indicate that more attention should be given to the metabolic potency of specific microbial groups in the colon lumen towards contaminants. Of course, colon microbiota are not solely presenting health risks towards their host. There are also microbial groups that exert beneficial effects such as the synthesis of fermentation products that provide energy to the colon epithelium (Cummings and Englyst, 1987), the stimulation of the gut immune system (Salminen et al., 1998), the synthesis of vitamins K and B (Conly and Stein, 1992) and the colonisation resistance against exogenous pathogens (Hopkins and Macfarlane, 2003).

The balance between these health-promoting and hazardous microbial groups and their metabolic reactions is rather delicate. Research suggests that the GI microbiota can be beneficially affected by means of a well-balanced diet and consumption of functional foods such as pro- and prebiotics (Gibson and Roberfroid, 1995; Collins and Gibson, 1999; Gibson and Williams, 1999). Probiotic food products contain live lactic acid producing bacteria such as lactobacilli, bifidobacteria and streptococci. If these health-promoting bacteria are still active when reaching the large intestine, they may support the growth of indigenous beneficial bacteria from the gut and suppress the colonization of pathogens by lowering the intestinal pH and producing bacteriotoxins (Fooks et al., 1999). A successful proliferation of probiotic bacteria in the human colon however depends on their survival through the acidic stomach environment, the membrane damaging effects from small intestinal bile salts and substrate- and niche competition with other intestinal microorganisms. Therefore, the concept of prebiotics has been developed.

Prebiotics are indigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a number of health-promoting colon bacteria such as lactobacilli and bifidobacteria (Gibson and Roberfroid, 1995). Of all the possible prebiotics, the effects of inulin type fructooligosaccharides (FOS) have been investigated the most. FOS are a mixture of indigestible but fermentable β-D-fructans with variable degree of polymerization. The average dietary intake of inulin by humans is estimated to be 1-4 g d^{-1} (Van Loo et al., 1995). Inulin would typically alter the composition of the human colon microbiota towards a predominance of bifidobacteria which can readily metabolise inulin, by which they have a nutritional advantage over other colon microbiota (Gibson et al., 1995; Brighenti et al., 1999; Hopkins and Macfarlane, 2003). In this way, the production of hazardous metabolites from bacteroides, clostridia or coliforms would decrease through the addition of inulin.

It this study, we investigated the prebiotic effects from native chicory inulin in combination with its inhibitory properties towards the microbial bioactivation of PAHs towards metabolites with estrogenic properties. Molecular analysis techniques and conventional methods were used to evaluate how inulin affects the composition and fermentation activity of *in vitro* cultured colon microbiota. The estrogen receptor bioassay was used to study the influence of inulin addition towards the microbial bioactivation potency of PAHs. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME), which harbours a microbial community resembling that from the human colon both in fermentation activity as in composition (Molly et al., 1993), was operated at full scale.

Materials and methods

SHIME run at full scale – inulin supplementation

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is a dynamic model of the human gastrointestinal tract (Molly et al., 1993; De Boever et al., 2000). It consists of 5 double-jacketed vessels maintained at a temperature of 37 °C, respectively simulating the stomach, small intestine, ascending colon, transverse colon and descending colon, with a total retention time of 76 h (Figure 6.1). The colon vessels harbour a mixed microbial community and pH controllers (pH controller R301, Consort, Turnhout, Belgium) maintain the pH in the range 5.6-5.9, 6.2-6.5 and 6.6-6.9 in the ascending colon, transverse colon and descending colon, respectively. There is no gas exchange between the different vessels and the headspace of the culture system was flushed twice a day for 15 min with N2 to ensure anaerobic conditions. The growth medium for the microbial inoculum consisted of a carbohydrate-based medium containing arabinogalactan (1 $g.L^{-1}$), pectin (2 $g.L^{-1}$), xylan (1 $g.L^{-1}$), starch (4.2 $g.L^{-1}$), glucose (0.4 $g.L^{-1}$), yeast extract (3 $g.L^{-1}$), peptone (1 $g.L^{-1}$), mucin (4 $g.L^{-1}$) and cysteine (0.5 $g.L^{-1}$). The pH of the medium was 5.5. Detailed information on the medium can be found in Molly et al. (1993).

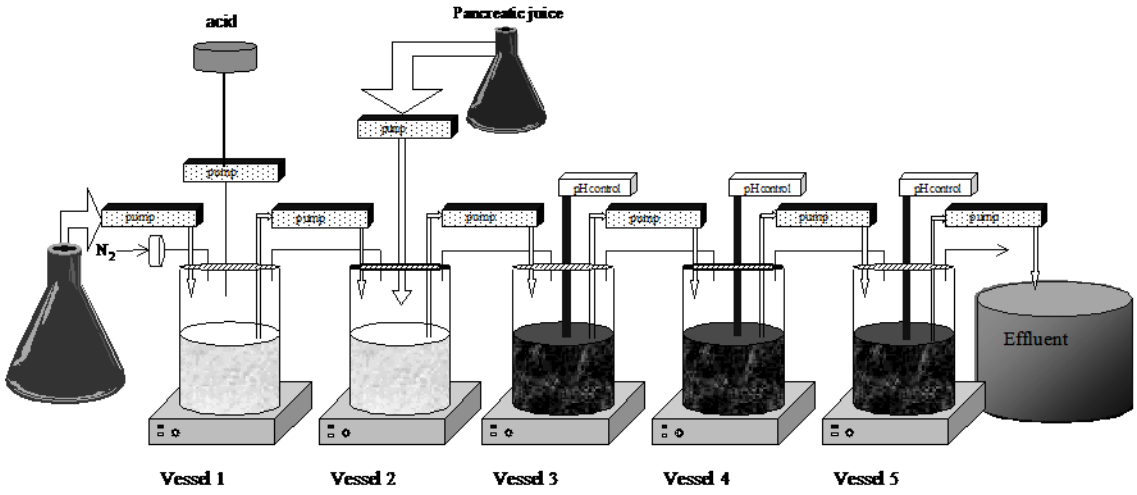


Figure 6.1 Schematic representation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Vessels 1 to 5 respectively simulate conditions from the stomach, small intestine, colon ascendens, colon transversum and colon descendens. (Source: De Boever et al., 2000)

At the beginning of the experiment, the last three vessels were inoculated with a pooled fecal sample of three adult volunteers who had no history of antibiotic treatment in the last year. During the start-up period, the normal nutritional medium was supplemented to the reactor, which enabled the microbial community to adapt themselves to the nutritional and physicochemical conditions that prevail in the different colon vessels (Molly et al., 1993). After two weeks, the treatment period was initiated, which lasted for five weeks. The nutrition for the treatment period consisted of the normal compounds as described above, except that the amount of starch in the medium was completely replaced by native chicory inulin (Fibruline Instant, COSUCRA, Warcoing, Belgium). This commercial inulin product has a dry matter of 96% and contains, on dry matter, 92% FOS with an average polymerisation degree of 10 and 8% free sugars. The dose of inulin to the SHIME reactor was 2.5 g d^{-1}, which was equivalent to a human dose of 5 g inulin d^{-1}. As the inulin replaced the starch concentration in the medium, the amount of available carbohydrates for the microorganisms stayed the same throughout the entire SHIME run. After the treatment period, a final control period concluded the run, to see whether the metabolic parameters and/or microbial concentrations evolved towards their initial values from the start-up period. This period lasted for 2 weeks and the inulin compound was again replaced by starch in the medium.

Chemical analysis

Short chain fatty acids (SCFA). Liquid samples were collected and frozen at -20 °C for subsequent analysis. The SCFA were extracted from the samples with diethyl ether and determined with a Di200 gas chromatograph (GC; Shimadzu, 's-Hertogenbosch, The Netherlands). The GC was equipped with a capillary free fatty acid packed column (EC-1000 Econo-Cap column (Alltech, Laarne, Belgium), 25 m×0.53 mm; film thickness 1.2 µm), a flame ionization detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as carrier gas at a flow rate of 20 mL min^{-1}. The column temperature and the temperature of the injector and detector were set at 130 °C and 195 °C, respectively.

Ammonia. Using a 1026 Kjeltec Auto Distillation (FOSS Benelux, Amersfoort, The Netherlands), ammonium in the sample was liberated as ammonia by the addition of an alkali (MgO). The released ammonia was distilled from the sample into a boric acid solution. The solution was back-titrated using a 665 Dosimat (Metrohm, Berchem, Belgium) and 686 Titroprocessor (Metrohm).

Lactate. Lactate concentrations were measured by dissolving 10 µL of sample in 990 µL lactate reagent (Sigma, Bornem, Belgium). Absorbance at 540 nm after 10 min incubation was read with an UVIKON 930 spectrophotometer.

Enzyme analysis. The samples were centrifuged at 10 000×g for 10 min. Cell free supernatant (100 µL) was pipetted into a 96-well plate, with 100 µL of a 5.0 mM solution of substrate, prepared in a 0.1 mM phosphate buffer (pH 6.5). The substrates (Sigma, Bornem, Belgium) used were p-nitrophenyl-β-galactopyranoside, p-nitrophenyl-β-glucuronide for β-galactosidase and β-glucuronidase. The plates were incubated at 37°C and the absorbance at 405 nm was read after 30 min with a Biokinetics EL312e multi-well reader (Bio-Tek Instruments Europe, Spijkenisse, The Netherlands). The amount of p-nitrophenol released was measured based on a standard curve of p-nitrophenol. The results were expressed in µmol p-nitrophenol released (mL min)$^{-1}$.

Microbial analysis

Plate counting. The analysed bacterial groups and the used media, purchased from Oxoid (Hampshire, UK), are indicated in Table 6.1. Liquid samples were withdrawn from the culture system and serially diluted in saline solution (8.5 g NaCl L^{-1}). Three plates were inoculated with 0.1 mL sample of three dilutions, and incubated at 37°C (43°C for E. coli) using conditions given in Table 6.1. Anaerobic incubation of plates was performed in jars with a gas atmosphere (84% N_2, 8% CO_2, and 8% H_2) adjusted by the Anoxomat 8000 system (Mart, Sint-Genesius-Rode, Belgium).

Table 6.1. Analyzed microbial groups together with the isolation media used and the incubation conditions

Bacterial group	Medium	Condition	Time
			h
Total aerobes	Brain Heart Infusion agar	Aerobic	24
Total anaerobes	Brain Heart Infusion agar	Anaerobic	72
Bifidobacteria	Raffinose *Bifidobacterium* agar	Anaerobic	72
Lactibacilli	Rogosa agar	Anaerobic	72
Fecal coliforms	Mc. Conkey agar	Aerobic	24
Enterococci	*Enterococcus* agar	Aerobic	48
Staphylococci	Mannitol Salt agar	Aerobic	48
Clostridia	Tryptose Sulfite Cycloserine agar	Anaerobic	24
Fungi	Martin agar	Aerobic	72

PCR-DGGE. The protocol for total DNA extraction from the SHIME samples was based on Boon et al. (2000). Three microbial groups of the colon were analysed: general bacteria, bifidobacteria and lactobacilli. A nested PCR approach (Boon et al., 2002) was used to amplify the 16S ribosomal RNA genes of the bifidobacteria and lactobacilli. In brief, one µl of the DNA was amplified using the primers BIF164f-BIF662r (Satokari et al., 2001) and SGLAB0158f-SGLAB0667 (Heilig et al., 2002), respectively for bifidobacteria and lactobacilli. When the first PCR round gave a clearly visible band, a second amplification round with forward primer P338F (with a GC-clamp of 40 bp) and reverse primer P518r was used (Muyzer et al., 1993). The 16S rDNA of all bacteria was amplified by applying primers P338F with GC-clamp and P518r on total extracted DNA.

DGGE (Denaturing Gradient Gel Electrophoresis) based on the protocol of Muyzer et al. was performed using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). On each gel, a home made marker of different PCR fragments was loaded, which was required for processing and comparing the different gels (Boon et al., 2002). The polyacrylamide gels were made with denaturing gradient ranging from 45 to 60 % (Boon et al., 2000). The electrophoresis was run for 16 hours at 60°C and 38 V. Staining and analysis of the gels was performed as described previously (Boon et al., 2000). The normalization and analysis of DGGE gel patterns was done with the BioNumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The statistical comparison of the DGGE patterns on the same gel was done with the GelCompar software 4.1 (Applied Maths, Kortrijk, Belgium). The calculation of the similarity matrix was based on the Pearson correlation coefficient. Clustering algorithm of Ward was used to calculate dendrograms (Ward, 1963).

DNA sequencing. 16S rDNA gene fragments were cut out of the DGGE gel with a clean scalpel and added to 50 µl of PCR water. After 12 hours of incubation at 4°C, 1 µl of the PCR water was reamplified with primer set P338F and P518r. Five µl of the PCR product was loaded on a DGGE gel (see above) and if the DGGE pattern only showed 1 band, it was sent out for sequencing. DNA sequencing of the ca. 180 bp fragments was carried out by ITT Biotech-Bioservice (Bielefeld, Germany). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Boon et al., 2002).

Nucleotide sequence accession numbers. Nucleotide sequences for bands Bif1, 2 and 3 and Bac1 have been deposited in GenBank database under accession numbers AY647970, AY647971, AY647972 and AY647973, respectively.

Real-time PCR. The real-time PCR was based on the principle of Heid et al. (1996). For quantification of bifidobacteria by real-time PCR, amplification was performed in 25-µl reaction mixtures by using buffers supplied with the qPCR™ Core Kit for Sybr™ Green I as described by the suppliers (Eurogentec, Liège, Belgium) in MicroAmp Optical 96-well reaction plates with optical caps (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). Primers BIF164f-BIF163r (Satokari et al., 2001) for 16S ribosomal RNA genes were used for the quantification of bifidobacteria at a concentration of 1 µM. PCR temperature program was as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 94°C for 1 min, 62°C for 1 min and 60°C for 1 min. The template DNA in the reaction mixtures was amplified (n=3) and monitored with an ABI Prism SDS 7000 instrument (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). DNA was extracted from a 6.4 x 107 CFU mL^{-1} culture of Bifidobacterium breve (LMG11042) (Kowalchuk et al., 1998). Standard curves were constructed after real-time PCR amplification of four different DNA concentrations (n=4) ranging from 1.28 x 107 to 1.28 x 104 cell equivalents/well. The standard curve had a R^2 value of 0.99 and the slope was -4.3.

Microbial PAH bioactivation

PAH incubation. During the start-up period and the treatment period of the SHIME run, weekly samples of 50 mL were taken from the different colon compartments of the SHIME reactor to perform incubation experiments in batch with benzo(a)pyrene as model PAH compound. Benzo(a)pyrene (40 µM) was incubated in the colon suspension (O_2=0.12 mg L^{-1}; Eh = -180 mV) at 37 °C for 72 h. No samples were incubated from the control period after inulin supplementation was stopped.

Sample treatment. Upon incubation, the digest mixtures were extracted using liquid/liquid extraction with ethylacetate in a 1:1 ratio. The ethylacetate fraction was subsequently put in a rotavapor to remove most of the solvent. The remainder of the solvent was removed under a gentle stream of nitrogen gas and finally replaced by dimethylsulfoxide (DMSO) which is a suitable solvent to use in the estrogen receptor bioassay.

Bioassay. The estrogen receptor test was used as previously described (Van de Wiele et al., 2004c). Basically, this test follows a modified protocol from De Boever et al. (2001) that was based on the protocol developed by Routledge and Sumpter

(1996). The latter researchers transformed *Saccharomyces cerevisiae* with the human estrogen receptor (ERα) gene, together with an expression plasmid containing estrogen responsive elements and the *lacZ* reporter gene (encoding the enzyme β-galactosidase). The expression of β-galactosidase is triggered by test chemicals, which upon binding to the estrogen receptor induce the conformational change necessary for binding of the receptor/ligand dimer to the responsive elements. This β-galactosidase activity is quantified at 540 nm by the conversion of the chromogenic substance chlorophenol red-β-D-galactopyranoside into chlorophenol red. The bioassay response is calculated as the absorbance at 540 nm divided by the optical density at 630 nm $(A_{540}/A_{630})_{net}$. Estrogenic activity of the samples was expressed as percentage equivalence to 6.96 nM 17α-ethynyl-estradiol (EE2) which elicited a 100 % response in the estrogen receptor bioassay (De Boever et al. 2001). To make sure that background signals from the gastrointestinal suspension as such did not interfere with the detection of estrogenic signals in the bioassays, corrections were made in a set of negative control experiments as previously described (Van de Wiele et al., 2004c).

Statistical analysis

Statistical analysis of the fermentation and microbial parameters was performed on samples taken over time once stable conditions during the subseqent start-up, treatment and control periods were obtained. It was not feasible to perform replicate SHIME runs since one run with the SHIME reactor takes more than 3 months. An unpaired 2-tailed t-test was used to compare averages.

Results

Inhibitory effects from inulin towards PAH bioactivation

To evaluate the effects from inulin towards the microbial conversion of PAHs to estrogenic metabolites, the bioactivation potency of the colon microbiota was checked by incubating benzo(a)pyrene at a concentration of 40 µM in mixed microbial suspensions retrieved from the different colon compartments of the SHIME reactor. Bioactivation was evaluated by measuring the estrogenicity of the samples using the yeast estrogen assay. Incubated samples from the start-up period were compared with incubated samples from the inulin treatment period (Figures 6.2, 6.3 and 6.4).

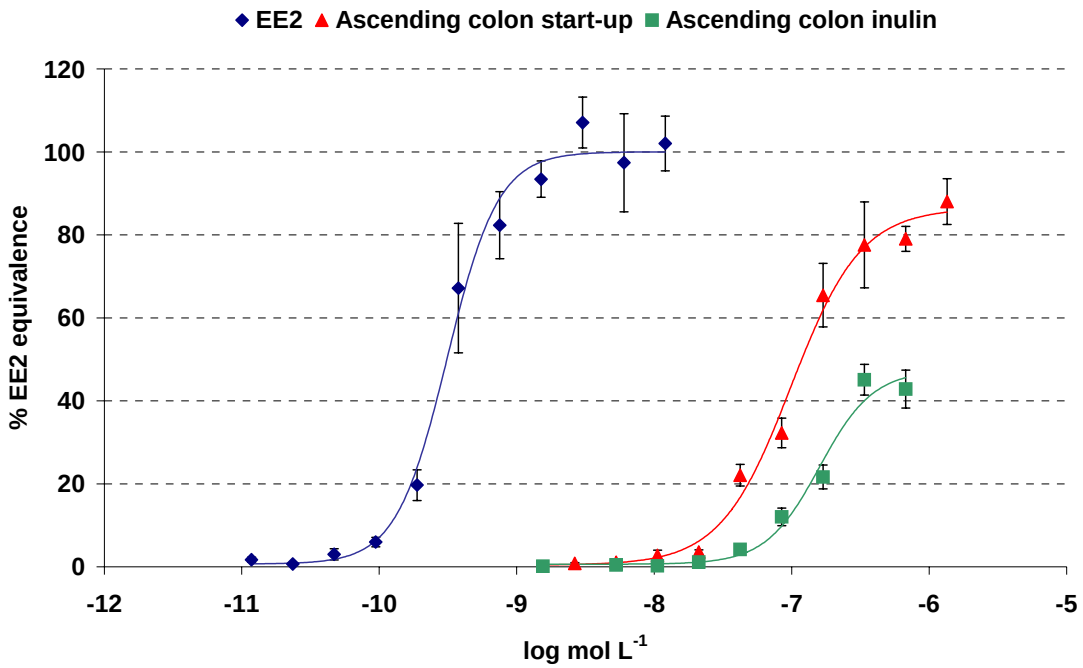


Figure 6.2 Estrogen response of benzo(a)pyrene incubated microbial suspension, sampled from the ascending colon compartment (n = 4)

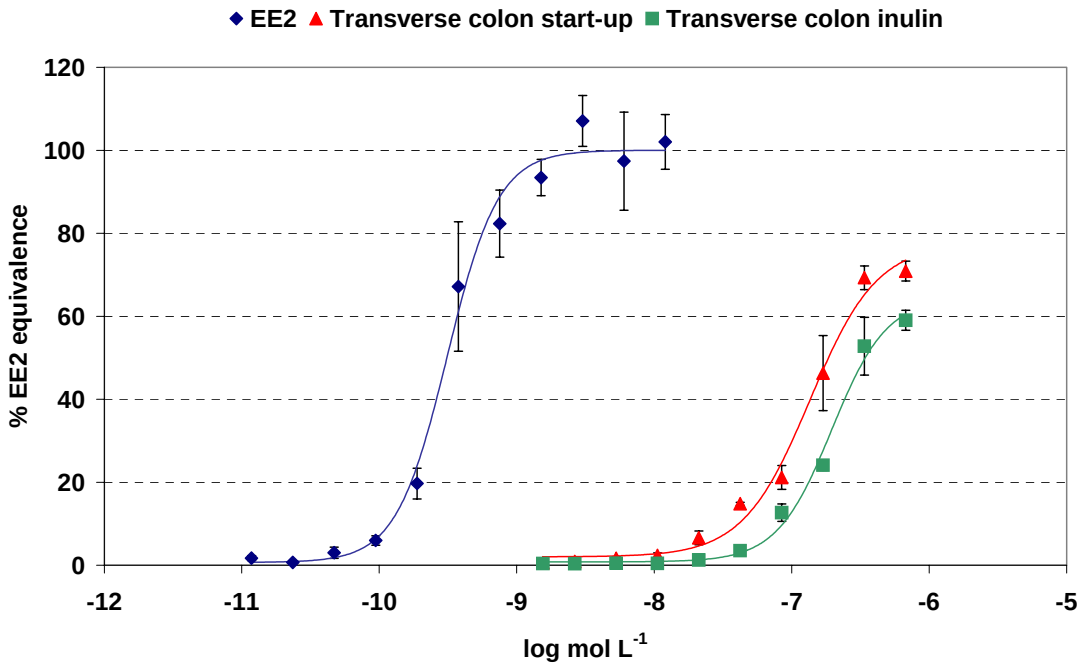


Figure 6.3 Estrogen response of benzo(a)pyrene incubated microbial suspension, sampled from the transverse colon compartment (n = 4)

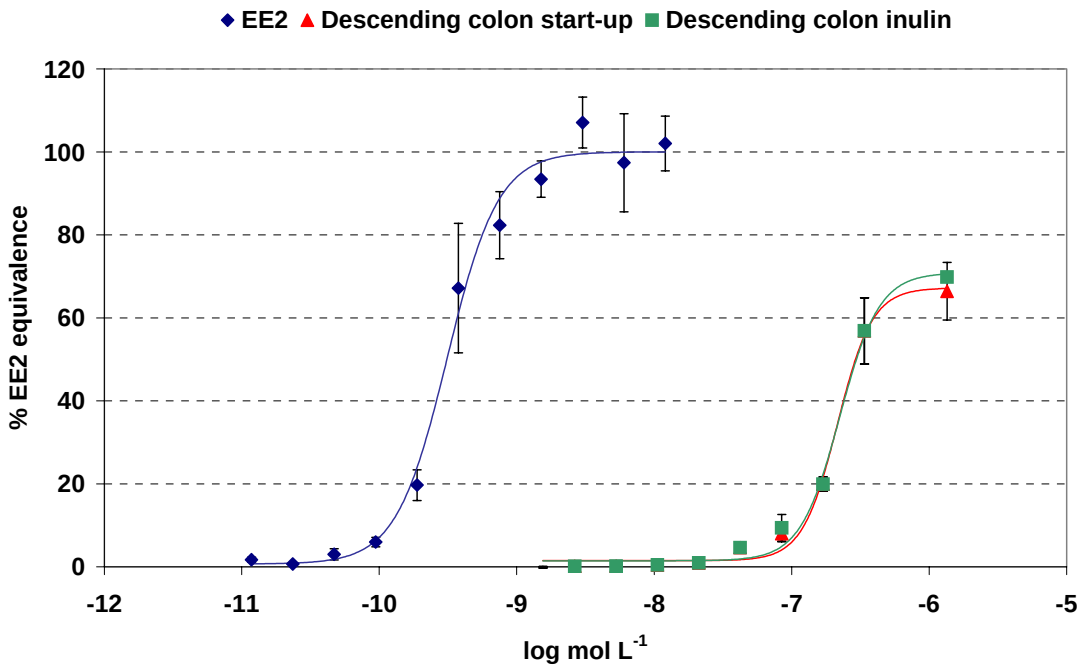


Figure 6.4 Estrogen response of benzo(a)pyrene incubated microbial suspension, sampled from the descending colon compartment (n = 4)

Confirming the results from Chapter 5, benzo(a)pyrene incubated colon samples elicited an estrogenic response after 72 h, whereas non-incubated samples elicited no positive response (data not shown). The highest estrogenicity was observed in the ascending colon (86±3.4% EE2 equivalence), followed by the transverse (78±5.2%) and descending colon (67±2.5%). Interestingly, incubation of benzo(a)pyrene with samples that were taken during the period of inulin supplementation showed a significantly lower estrogen response. The difference with the start-up period was most obvious in the ascending colon samples (47±4.3%), followed by the transverse colon samples (64±4.3%). No difference in estrogen response was seen in the descending colon samples, indicating the specificity of the chemopreventive effect of inulin in the ascending colon. The effect from inulin supplementation towards the estrogenicity of the samples was also evidenced by shifts in the EC_{50}-values when inulin was added.

The estrogen bioassay results clearly indicate the effect from inulin supplementation towards the estrogenic response of samples from the respective colon compartments that were incubated with benzo(a)pyrene. To explain these inhibitory effects from inulin, a possible relationship with the well-known prebiotic effects from inulin was evaluated. Therefore, both the metabolic activity from the microbiota in the respective colon compartments of the SHIME reactor, as the microbial community composition were investigated.

Metabolic activity

Replacement of starch by a metabolic equivalent amount of chicory inulin in the nutrition of the gastrointestinal simulator changed the microbial fermentation pattern in all colon vessels towards a more saccharolytic metabolism. This metabolic shift was already observed after 1 week of inulin supplementation (data not shown) and eventually resulted in a significant ($p<0.01$) increase in short chain fatty acid (SCFA) production with 44, 23 and 33% in the ascending, transverse and descending colon at the end of the 5 week treatment period (Table 6.2). The higher SCFA concentrations primarily originated from an increased production of propionate and butyrate, whereas no significant changes in concentrations of acetate or branced fatty acids were observed. During inulin administration, ammonia concentrations significantly decreased in the ascending colon vessel, whereas no significant changes in ammonia production were observed in the other colon vessels. As for lactate production, no significant changes were detected upon administration of inulin. In the control period, inulin was removed from the nutrition for the SHIME reactor and again replaced by the original amount of starch (4.2 g L^{-1}). SCFA production again decreased with 26%, 30% and 28% in the ascending, transverse and descending colon and approached the initial levels from the start-up period (Table 6.2). This was primarily due to lower levels ($p<0.01$) of propionate and butyrate. The metabolic shift during the control period was also apparent from a higher ammonia production (33%) and a decrease in lactate production in the ascending colon vessel and the increase in lactate concentrations in the transverse colon vessel. Enzymatic activities of β-galactosidase and β-glucuronidase were monitored in the respective colon compartments and did not change significantly during the entire SHIME run (data not shown).

Microbial community analysis

Plate counting. Using selective growth media, analysis of the microbial suspension from the SHIME colon compartments revealed that inulin administration had limited effects on the overall composition of the microbial community. Concentrations of the beneficial microbial groups, bifidobacteria and lactobacilli, increased in all colon vessels during the first weeks of inulin supplementation (data not shown), yet this increase only became significant during the last two weeks of the treatment period. For the transverse and descending colon compartments, lactobacilli concentrations were 1.5 log CFU higher ($p<0.01$) than the initial levels from the start-up period (Table 6.3). Significantly higher bifidobacteria concentrations ($p<0.05$) were observed in the ascending colon after 5 weeks of inulin supplementation. A limited

decrease in staphylococci concentrations ($p<0.05$) was observed in the descending colon whereas fecal coliforms were inhibited both in the transverse colon ($p<0.05$) and descending colon ($p<0.01$) (Table 6.3). During the control period, starch again replaced inulin in the nutrition of the SHIME reactor. This resulted in lower lactobacilli concentrations in the transverse colon and lower bifidobacteria concentrations in the ascending colon, whereas no other significant changes compared to the startup period were found.

Table 6.2 Concentration of short-chain fatty acids, NH4+ and lactate measured in vessels 3, 4 and 5 of the SHIME during the start-up (n = 14), treatment (n = 13) period with inulin after 5 weeks and control period (n = 6). Data are means ± SD,

	Parameter	Asc. Colon	Trans. Colon	Desc. Colon.
			µmol/mL suspension	
Start-up	Acetic acid	21.2 ± 5.4	30.4 ± 2.5	32.7 ± 3.6
	Propionic acid	7.0 ± 2.1	10.6 ± 0.5	10.3 ± 1.3
	Butyric acid	7.7 ± 4.3	14.5 ± 3.4	12.9 ± 5.3
	Other acids	1.1 ± 0.6	1.9 ± 0.2	2.3 ± 0.7
	Total SCFA	36.9 ± 5.9	57.4 ± 2.8	58.2 ± 9.4
	Ammonium	15.6 ± 0.9	23.4 ± 2.9	26.2 ± 3.5
	Lactate	1.35 ± 0.49	0.13 ± 0.04	0.10 ± 0.02
Treatment	Acetic acid	19,9 ± 2,9	28,1 ± 4,5	33,9 ± 4,0
	Propionic acid	17,6 ± 1,7**	21,3 ± 2,2**	21,6 ± 2,1**
	Butyric acid	14,3 ± 2,1**	19,3 ± 1,7**	19,3 ± 1,2**
	Other acids	1,4 ± 0,3	2,0 ± 0,4	2,9 ± 0,4
	Total SCFA	53,3 ± 4,9**	70,7 ± 5,6**	77,6 ± 4,1**
	Ammonium	11,5 ± 1,7**	21,1 ± 2,7	24,3 ± 1,9
	Lactate	0,71 ± 0,19	0,13 ± 0,03	0,13 ± 0,05
Control	Acetic acid	19.1 ± 9.5	25.3 ± 2.1**	30.5 ± 3.0
	Propionic acid	7.6 ± 2.3°°	11.9 ± 1.8°°	12.2 ± 2.6°°
	Butyric acid	11.5 ± 5.3°°	10.8 ± 4.3°°	10.7 ± 4.0°°
	Other acids	1.4 ± 0.6	1.5 ± 0.5	2.5 ± 0.6
	Total SCFA	39.6 ± 3.8°°	49.6 ± 4.5°°**	55.9 ± 9.4°°
	Ammonium	15.3 ± 0.8°°	21.8 ± 3.0	23.9 ± 4.4
	Lactate	0.12 ± 0.02°°	0.40 ± 0.01°°**	0.12 ± 0.03

* Significantly different from the start-up period, $p \leq 0.05$; ** $p \leq 0.01$,

° Significantly different from treatment period, $p \leq 0.05$; °° $p \leq 0.01$

Table 6.3 Microbial counts in the colon vessels of the SHIME reactor during 2 weeks of start-up (n = 5), 5 weeks of treatment with inulin at normal concentration (n = 5) and 2 weeks of control period (n = 3). Data are means ± SD,

	Microbial group	Ascending Colon	Transverse Colon	Descending Colon
		Log CFU/mL		
Start-up	Aerobe	8,27 ± 0,15	8,34 ± 0,13	8,31 ± 0,52
	Anaerobe	8,18 ± 0,14	7,79 ± 0,81	7,55 ± 0,75
	Lactobacilli	5,57 ± 0,69	5,42 ± 0,75	5,01 ± 0,83
	Enterococci	6,98 ± 0,25	6,91 ± 0,20	6,81 ± 0,14
	Fecal coliforms	6,66 ± 0,40	7,00 ± 0,40	7,32 ± 0,28
	Bifidobacteria	6,26 ± 0,22	6,83 ± 0,84	6,75 ± 0,62
	Staphylococci	7,15 ± 0,17	6,59 ± 0,42	6,75 ± 0,08
	Fungi	3,43 ± 0,22	3,20 ± 0,60	2,80 ± 0,14
	Clostridia	8,12 ± 0,22	8,13 ± 0,24	7,72 ± 0,29
Treatment	Aerobe	7,82 ± 0,96	8,40 ± 0,58	7,62 ± 0,26
	Anaerobe	7,98 ± 0,45	8,15 ± 0,33	8,35 ± 0,34
	Lactobacilli	6,39 ± 0,21	6,91 ± 0,13**	6,64 ± 0,05**
	Enterococci	6,58 ± 0,23	6,68 ± 0,22	6,57 ± 0,25
	Fecal coliforms	6,28 ± 0,30	6,29 ± 0,29*	6,33 ± 0,37**
	Bifidobacteria	6,93 ± 0,29*	6,57 ± 0,24	6,75 ± 0,56
	Staphylococci	6,70 ± 0,28	6,53 ± 0,05	6,43 ± 0,20*
	Fungi	3,36 ± 0,44	2,40 ± 0,14	2,40 ± 0,14
	Clostridia	7,53 ± 0,53	8,20 ± 0,94	8,01 ± 0,61
Control	Aerobe	8,01 ± 0,53	8,06 ± 0,42	8,08 ± 0,48
	Anaerobe	8,18 ± 0,47	7,80 ± 0,65	7,66 ± 0,93
	Lactobacilli	6,77 ± 0,18	6,48 ± 0,04°	6,68 ± 0,14
	Enterococci	6,78 ± 0,61	6,21 ± 0,89	6,47 ± 0,06*
	Fecal coliforms	6,45 ± 0,47	6,54 ± 0,22	6,56 ± 0,40*
	Bifidobacteria	5,69 ± 0,22°	5,57 ± 0,56	5,39 ± 0,34
	Staphylococci	6,56 ± 0,69	6,40 ± 0,53	6,12 ± 0,71
	Fungi	3,15 ± 0,40	- ± -	- ± -
	Clostridia	7,81 ± 0,52	7,23 ± 1,07	7,71 ± 0,05

* Significantly different from the start-up period, $p \leq 0.05$; ** $p \leq 0.01$,

° Significantly different from treatment period, $p \leq 0.05$; °° $p \leq 0.01$

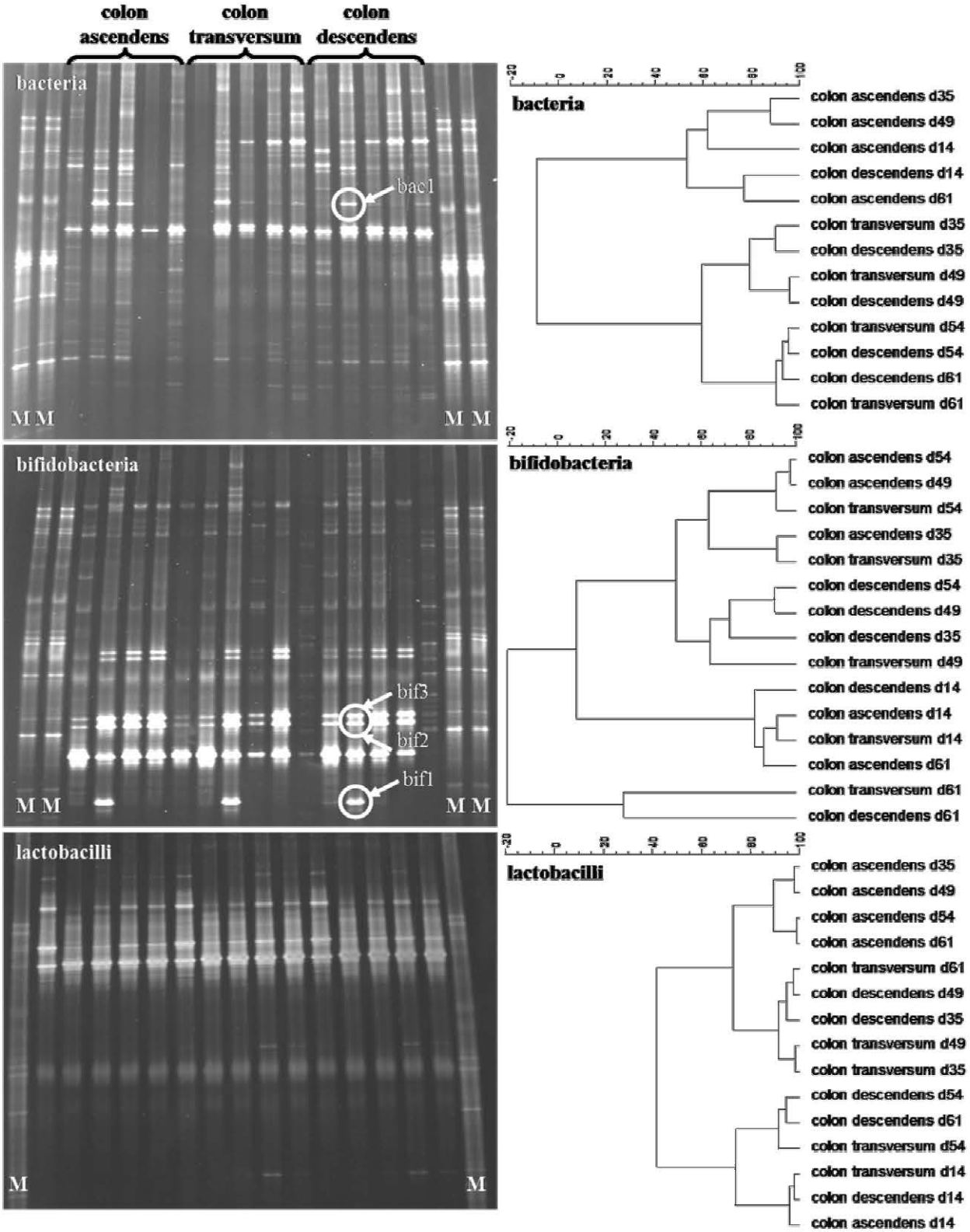
Microbial fingerprinting. PCR-DGGE was used as a molecular fingerprinting technique to monitor qualitative changes in the composition of the microbial community from the three colon compartments throughout the SHIME run. Samples from the start-up period were taken at day 14, just before the supplementation of inulin began. Samples from the treatment period were taken at day 35 and day 49, respectively after 3 and 5 weeks of inulin supplementation. Finally, samples from the control period, during which no inulin was supplemented, were taken at day 54 and 61. Thus, for the three colon compartments, a total of 15 samples was collected and DGGE analysis was performed for general bacteria, bifidobacteria and lactobacilli.

The global fingerprint for general bacteria showed that all samples from the ascending colon clustered in a separate group and that most samples from the transverse and descending colon clustered together in another group (Figure 6.5). Both within the ascending colon group as the transverse/descending colon group, the influence of inulin addition was observed by the separate clustering of the treatment samples d35 and d49 and the control samples d54 and d61 (Figure 6.5). Although this inulin effect was slightly apparent within each colon compartment, the dominant factor for clustering was the colon compartment itself, from which the samples were taken. This roughly corresponds to the limited variations in microbial populations that were observed using conventional plating techniques. This was in contrast to clustering analysis of DGGE patterns for the bifidobacteria. For this bacterial group, samples did not cluster according to the colon compartment of origin, but to the time point at which they were taken (Figure 6.5). All colon samples from the treatment period (d35 and d49) clustered together with the first colon samples from the control period (d54). For all three colon vessels, a new band strongly appeared at day 35, after three weeks of inulin supplementation.

Samples from the start-up period (d14) and the second control sample from the descending colon formed a second cluster, whereas the control samples at d61 for the ascending and transverse colon clustered separately. For lactobacilli, the clustering pattern was roughly comparable to that for bifidobacteria. All samples from the start-up period (d14) formed a first cluster together with several samples from the control period (d54 or d61), whereas all samples from the treatment period (d35 and d49) and some of the control period were grouped in a second cluster. At higher similarity values, two additional clusters were distinguished, a first one formed by ascending colon samples from the treatment (d35 and d49) and control period (d54 and d61) and a second one formed by several samples from the transverse and descending colon for the treatment and control period.

Sequencing. Based on the DGGE fingerprint analysis of the colon microbial community, several shifts in bands or changes in band intensity were observed. To identify the bacterial species that were responsible for those changes, DNA fragments from bands of interest were excised from the DGGE gel, isolated and finally sequenced. Four bands were succesfully sequenced. DNA fragment bands marked "bif1" on the bifidobacteria DGGE gel showed 95% similarity (141 out 147 bases) to an uncultured bacterium isolated from mucosa associated bifidobacteria (AY267921). These bands strongly appeared during the first weeks of inulin supplementation, but then declined again in intensity. Two other bands – marked "bif2" and "bif3" – also got more intense during inulin supplementation and these bands respectively revealed similarity to *Bifidobacterium infantis* (96% similarity, 169 out of 176 bases, AY166531.1) and *Bifidobacterium longum* (96% similarity, 170 out of 177 bases, AY166538.1). For the bacterial DGGE pattern too, a remarkable change was observed during inulin administration, with one band marked "bac1" getting more intense in all colon vessels. The DNA sequence of this band showed 93% similarity (178 out 192 bases, AF371889) to an uncultured bacterium from the GI tract, belonging to the genus of Prevotella and was much less intense during the start-up and control period.

Figure 6.5 DGGE fingerprint patterns for general bacteria, bifidobacteria and lactobacilli. "M" indicates marker lanes on the gel. Between the markers, samples are organized as follows: colon ascendens - d14, d35, d49, d54, d61; colon transversum - d14, d35, d49, d54, d61; colon descendens - d14, d35, d49, d54, d61. Samples d14 were taken during the start-up period, samples d35 and d49 were taken during the treatment period, whereas samples d54 and d61 were taken during the control period. Samples from the ascending colon d54 and transverse colon d14 were not taken up in the clustering analysis since the amount of loaded PCR product on the gel was too little.


colon ascendens
colon transversum
colon descendens
bacteria
bac1
M M
M M
bifidobacteria
bif3
bif2
bif1
M M
M M
lactobacilli
M
M
bacteria
colon ascendens d35
colon ascendens d49
colon ascendens d14
colon descendens d14
colon ascendens d61
colon transversum d35
colon descendens d35
colon transversum d49
colon descendens d49
colon transversum d54
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bifidobacteria
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colon transversum d35
colon descendens d54
colon descendens d61
colon transversum d54
colon transversum d14
colon descendens d14
colon ascendens d14

Real-time PCR. From the conventional culture based techniques, we observed a significant bifidogenic effect upon inulin supplementation. However, this effect was only seen in the ascending colon (0.7 log CFU increase). Yet, DGGE analysis showed that samples from all colon vessels clustered together during the treatment period. Therefore, we used real-time PCR as a cultivation independent method to quantify the number of bifidobacteria in samples from all colon compartments at the end of the start-up, treatment and control periods. We noted an overall higher bifidobacteria concentration with real-time PCR compared to those obtained using conventional plating techniques. The real-time PCR data showed a strong bifidogenic effect in all three colon vessels ($p<0.01$), whereas conventional plate count data only showed this effect in the ascending colon (Table 6.4). The observed increase in bifidobacteria concentrations was not maintained till the end of the control period during which no inulin was supplemented ($p<0.01$).

Table 6.4 Bifidobacteria concentrations in the intestinal suspension from the colon vessels during start-up (n = 5), 5 weeks of treatment with inulin at doses of 2.5 g d^{-1}(n = 5) and control period (n = 3) [a]

		Plate count (log CFU/mL)	Real-time PCR (log CFU/mL) [b]
Asc. Colon	**Start-up**	6.26 ± 0.22	7.45 ± 0.12
	Treatment	6.93 ± 0.29*	8.05 ± 0.07**
	Control	5.69± 0.22	7.41 ± 0.04°°
Trans. Colon	**Start-up**	6.83 ± 0.84	7.34 ± 0.16
	Treatment	6.57 ± 0.24	8.46 ± 0.11**
	Control	5.57 ± 0.56	7.21 ± 0.02°°
Desc. Colon.	**Start-up**	6.75 ± 0.62	7.14 ± 0.29
	Treatment	6.75 ± 0.56	8.01 ± 0.08**
	Control	5.39 ± 0.34	7.60 ± 0.03*°°

[a] Data are means ± SD,

* Significantly different from the start-up period, $p \leq 0.05$; ** $p \leq 0.01$, ° Significantly different from treatment period, $p \leq 0.05$; °° $p \leq 0.01$

[b] Real-time PCR values are averages of 3 replicates

Discussion

In this study, we have shown that inulin may purport not only prebiotic effects towards an *in vitro* cultured microbial community of the human colon, but also that it acts as a chemopreventive agent towards the microbial bioactivation of PAHs to compounds with estrogenic properties. The latter was evidenced by a lower estrogenicity from colon suspensions that were sampled from the SHIME reactor after 3 weeks of inulin treatment and that had been incubated with benzo(a)pyrene for 72 h. The chemopreventive effect from inulin was most apparent from ascending colon samples, whereas descending colon samples were not affected by inulin addition. These *in vitro* observations are the first for inulin as inhibitory compound towards PAH bioactivation by intestinal microbiota. The chemopreventive effects occurred after three weeks of inulin addition to the SHIME reactor at a dose of 2.5 g d^{-1}, which corresponds to an equivalent human dose of 5 g d^{-1}. This is a feasible human daily intake and well within the range of earlier reports testing the effects of inulin *in vitro* and *in vivo* Macfarlane et al., 1998; Rao, 2001). Similar inhibitory effects from inulin were previously reported for the microbial formation of hazardous heterocyclic aromatic amines (HAA), which resulted in a decreased mutagenicity (Shin et al., 2003). The chemopreventive effect of inulin can be quite strong as it significantly decreases the number of aberrant crypt foci, precursors from adenomas or carcinomas, in the rat colon (Reddy et al., 1997; Rowland et al., 1998). The chemopreventive effects from inulin towards overall mutagenicity in the colon, or in this study, the bioactivation of PAHs, can be explained by the prebiotic properties of inulin in the lumen of the GI tract.

In fact, successful application of inulin as prebiotic agent may have several consequences that are all related to health-promoting effects. Prebiotics promote the growth of health-beneficial microbial groups such as Bifidobacteria, thus keeping concentrations of bacteriodes, clostridia or coliforms at low levels (Gibson and Roberfoid, 1995) by the production of bacteriotoxins (Fooks et al., 1999). Also, the promotion of lactic acid producing bacteria in general is beneficial since these organisms possess antimutagenic and anticarcinogenic properties (Bodana and Rao, 1990). As a direct outcome of the stimulation of beneficial microbial groups, the increased production of SCFA by colon microbiota and the decreased production of ammonia and β-glucuronidase have direct health consequences as will be further discussed. An analysis of the prebiotic effects from inulin supplementation in this study may help explain the decreased estrogenicity of PAH incubated samples with inulin.

Moreover, the prebiotic effects may be related to the inhibition of hazardous health effects when toxic PAH metabolites make contact with the colon epithelium.

Firstly, administration of inulin to the nutrition of the SHIME reactor beneficially influenced the fermentation pattern of the colon microbiota towards a more saccharolytic environment with a significantly higher SCFA production, more in particular propionate and butyrate, and a lower ammonia production (Table 6.2). The 44% increase in SCFA production in the ascending colon seems uncommon since the treatment period entailed a replacement of starch by an equivalent amount of inulin and not an addition. The inulin degrading capacity from bifidobacteria would normally result in SCFA production levels that are comparable to the start-up period when an equivalent amount of starch was added. The additional SCFA production may possibly be explained by the additional bifidobacterial biomass, created by the bifidogenic effect from inulin. Additionally, other microbial groups in the colon suspension that are used to starch breakdown, may ferment alternative carbon sources from the nutrition medium to SCFA. This extra SCFA production needs clarification in further research. The shift towards propionate and butyrate corresponds to earlier observations by Uehara et al. (2001), who supplemented FOS to rats and saw a significant increase in concentration for these two SCFA. These observations do not directly point towards bifidogenic effects since bifidobacteria are acetate and lactate producers. Maybe other microbial groups such as *Megasphaera elsdenii* or *Roseburia* sp. can convert lactate or acetate to butyrate (Duncan et al., 2002; Hashizume et al., 2003). Similar processes in the SHIME reactor may explain the constant lactate and acetate concentrations during inulin treatment, whereas specific increases in bifidobacteria concentrations were seen. This needs to be addressed in further research. In general, increased SCFA synthesis creates a more acidic environment in the gut which is important *in vivo* in terms of colonization resistance against pathogens. Moreover, SCFA are important energy sources for the colonocytes and influence colonic function by stimulating water and sodium absorption and modulating motility (Cherbut et al., 1997). More specifically, butyrate also induces differentiation and together with propionate, it stimulates apoptosis of cancerous cells *in vitro* (Scheppach et al., 1995; Jan et al., 2002). In relation to the microbial bioactivation of benzo(a)pyrene in this study or for example mutagenic HCA formation in other studies (Shin et al., 2003), an increase in SCFA production is thus beneficial since it may decrease the risk of cancer development in the colon or rectum. Moreover, inulin administration also resulted in a significant decrease of ammonia levels in the ascending colon. This is important since ammonia can alter the morphology and intermediary metabolism of intestinal cells,

increase DNA synthesis and promote tumorigenesis (Ichikawa and Sakata, 1998). Lower proteolytic activities are therefore related to health-promoting effects.

SCFA and lactate analyses are important measures for intestinal metabolism, but in this research, they gave no direct information on bifidogenic effects. Therefore, we monitored β-galactosidase in the colon suspension of the SHIME reactor, since this enzyme is often related to the presence of bifidobacteria (Van Laere et al., 2000). Due to a large variability in the measurements, no significant changes in β-galactosidase levels were found in the colon compartments during inulin administration. We also monitored β-glucuronidase production, since the latter is often associated to hazardous processes in the gut. The reason for this is that microbial β-glucuronidase is involved in the intestinal hydrolysis of conjugated xenobiotics and may delay the excretion of harmful exogenous compounds (Weisburger, 1971). With respect to oral PAH exposure, as discussed in this dissertation, this may be important since a decreased β-glucuronidase production would increase the excretion of phase II PAH metabolites from the enterocytes or hepatocytes. However, no significant changes were detected in the absence or presence of inulin. Hence, from the metabolic perspective of inulin administration, we concluded that inulin administration had beneficial effects towards more SCFA production, but no direct evidence of bifidogenic effects was obtained.

With regards to effects towards the microbial community, Bifidobacteria have a nutritional advantage over other intestinal microorganisms due to their β-1,2-glycosidase activity, allowing them to metabolize FOS compounds from the inulin. Significant changes in microbial community composition were only observed after two weeks of inulin supplementation whereas metabolic changes were found within days. This can be explained by the faster adaptation of a microbial population towards their metabolism (RNA-based) than towards their community structure (DNA-based) (Boon et al., 2003). Plate count analysis of the microbial community structure revealed that bifidogenic effects in the ascending colon vessel and the increase in lactobacilli in the other colon vessels only became significant after three weeks of supplementation. This shows that the lower dose of 2.5 g d^{-1} from this study needs to be administered over a longer time frame to effectively induce and maintain beneficial effects. Single doses of inulin are therefore of no use. Besides the nutritional advantage that bifidobacteria may have from inulin over other intestinal microbiota, bifidobacteria together with lactobacilli also create a more acidic environment by the production of lactic acid, thus inhibiting the excessive growth of pathogens. Plate count data may support this hypothesis by the decreased concentration of opportunistic pathogens as E. coli and staphylococci (Table 6.3). Comparable inhibitory effects of inulin towards other pathogens as

Clostridium difficile were previously described (Macfarlane et al., 1998). The bifidogenic effect from inulin was however only observed in the ascending colon vessel. Interestingly, the chemopreventive effects of inulin treatment towards PAH bioactivation were also most apparent in the proximal colon compartments (Figure 6.3 and 6.4). This could be explained by the fact that the larger prebiotic effects towards bifidobacteria and lactobacilli in this colon compartment also result in a larger suppression of hazardous microbial groups such as Bacteriodes, Clostridia or coliforms. This would also decrease the number of hazardous metabolic reactions that these groups perform, including for instance the hazardous production of estrogenic PAH compounds. Moreover, sorption of hazardous compounds by lactic acid producing bacteria has been reported by Bolognani et al. (1997), thus reducing the mutagenicity in the gastrointestinal tract. Similar sorption phenomena by an increased number of Bifidobacteria may have affected the estrogenicity of the PAH incubated intestinal suspensions from the SHIME reactor.

Structural analysis of the colon microbiota using PCR-DGGE showed that administration of inulin during the treatment period significantly affected Bifidobacteria and Lactobacilli populations, whereas the overall microbial community kept relatively unchanged (Figure 6.5). Clustering of the Eubacteria DGGE patterns according to the colon vessel shows that the SHIME reactor harbours different microbial communities in the different colon vessels and that these populations are relatively unaffected by inulin. In contrast, clustering of the Bifidobacteria and Lactobacilli DGGE patterns showed that samples from the treatment period were grouped together. This confirms the data obtained with selective growth media and indicates the selectivity of inulin towards these health-promoting bacteria. The sequencing of the more intense DNA fragment bands on the DGGE gels pointed towards a previously uncultured *Bifidobacterium* species, *B. longum* and *B. infantis*. The latter organisms have been described to be beneficially affected by inulin derived substrates (Wang and Gibson, 1993; Mayer et al., 2003; Hopkis and Macfarlane, 2003) and are also used as probiotics. In contrast to the plate count data, the DGGE and sequencing analysis also showed that the bifidogenic effect was not restricted to the ascending colon alone, but was also visible in the distal colon vessels. To get a more precise idea of the bifidogenic effect in the different colon vessels, we used real-time PCR to quantify bifidobacteria.

In general, the higher bifidobacteria concentrations from real-time PCR analysis compared to plate count analysis (Table 6.4) may be explained by the fact that real-time PCR takes into acount all bifidobacteria including viable but non culturable organisms and inactive organisms. The bifidogenic effect of inulin in the SHIME reactor was strongly supported by real-time PCR data that showed a more pronounced increase in bifidobacteria, not only in the ascending colon vessel, but also in the other colon vessels. With conventional plating techniques, no significant bifidogenic effects were observed in the distal colon compartments. Analysis of real-time PCR results needs to carefully consider that changes in data may be attributed to stimulation of bacterial species with different copy number. However, the rrndb (ribosomal RNA operon copy number database) (Klapperbach et al., 2001) indicates that the copy number of the different bifidobacterial species is fairly constant, 3 or 4. Even if inulin promotes the growth of a species with a copy number more than the already present bifidobacteria, the difference attributed to this increase in copy number would be negligable to the difference of 1 log CFU mL^{-1} as observed in our study. To our knowledge, this is the first report indicating that real-time PCR is a more precise method by which more subtle differences in bifidobacteria populations can be detected, this in contrast to more variable results from conventional plate count techniques. This general bifidogenic effect from inulin did not correspond to the specific chemopreventive effect of inulin in the ascending colon. It is possible that an increased number of Bifidobacteria is able to successfully decrease PAH bioactivation reactions in the ascending colon by the inhibition of hazardous microbial groups. However, conditions in the distal colon compartments are less favourable to Bifidobacteria due to a higher pH and lower substrate availability. This would decrease its inhibitory effect towards pathogens and their associated hazardous metabolic activity.

In summary, our study showed beneficial effects from inulin treatment towards microbial metabolism and community composition. The inhibition of estrogenic PAH metabolite formation in the ascending colon can be considered as beneficial, since this would reduce the risks that bioactive PAHs may pose towards the colon epithelium. A complete elucidation of how this chemopreventive effect relates to the observed prebiotic effects requires further investigation of the responsible microbial groups and their associated metabolism. It is however interesting to remember that even at a fairly low dose of 2.5 g d^{-1}, significant chemopreventive effects were observed. Additionally, benefical metabolic effects were rapidly seen with an increase in SCFA synthesis towards butyrate and propionate and a decreased ammonia production. It should be

noted though that a prolonged supplementation period is needed to also observe beneficial effects at the microbial community level. Moreover, we found that the prebiotic effects from inulin were limited to the period of supplementation. In order to maintain these prebiotic effects and support the growth of beneficial bacteria, inulin should thus be continuously dosed. This study indicates the usefulness of advanced *in vitro* methods that mimick both the proximal as distal region of the colon, this in contrast to *in vivo* studies that often investigate fecal microbiota that are less representative of the colon lumen microorganisms.

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Relevance of this research

Importance of soil ingestion in HHRA

The potential exposure of soil contaminants to human or ecological receptors leads to significant health risks or ecological consequences that need careful monitoring in risk management processes. As discussed in **Chapter 1**, the latter are designed to remediate any adverse effects where impairment of the environment occurs and to minimize potential threats for human health. For human exposure, this necessitates an accurate and reliable human health risk assessment study which investigates the different exposure routes of the contaminants to the human body and the toxic effects of the bioavailable fraction. Human exposure to soil contaminants can be multiple and complex comprising several exposure pathways and exposure routes.

As part of the human health risk assessment process for these contaminated sites, exposure to contaminants via ingestion of soil is routinely estimated. Contaminated soils constitute a ubiquitous source of environmental pollutants and may be ingested as a result of recreation, gardening, consumption of locally grown foods and the ingestion of airborne contaminated dust (Swartjes, 2002). Children are normally thought to experience the highest risk due to their typical hand-to-mouth behavior which often leads to soil ingestion rates of 200, 500 mg and even several g d^{-1} (van Wijnen et al., 1990; Calabrese et al., 1997a; Calabrese et al. 1997b). However, also adults, more in particular construction workers, are often exposed to soil contaminants via the ingestion exposure route (Stanek et al., 1997). Reported soil ingestion rates for adults vary from 1 to 124 mg d^{-1} (Brunekreef et al., 1987; Calabrese et al., 1990). In brief, the default oral exposure factors that are proposed by EPA when performing site-specific soil screening evaluations are for adults and children 100 and 200 mg d^{-1} respectively (EPA, 1997).

To what extent the ingestion pathway contributes to the actual exposure of the human body to soil contaminants, is illustrated in Figure 7.1. Several European institutes have developed human exposure models which allow to estimate the extent of human exposure to soil contaminants, to predict associated toxicity levels and, if applicable, to indicate soil quality standards and e.g. treshold limit values (TLV). Comparison of the different models with one another for different model compounds reveals that soil ingestion is one of the predominant exposure routes to soil

contaminants (Swartjes, 2002). Only for benzene, as model compound for aromatic chemicals, direct soil ingestion is insignificant. Yet, the ingestion pathway is still well represented with 17% exposure due to crop consumption. Human exposure to soil-borne cadmium and benzo(a)pyrene, model compounds for heavy metals and PAHs respectively, primarily occurs through soil ingestion, whereas soil ingestion (37.2%) for atrazine as model compound for pesticides is equally important as crop consumption (38%).

These values indicate the need to perform research on the oral exposure to soil contaminants. When carrying out site-specific risk assessment studies, it needs to be taken into account that soil ingestion is an important contributor to the total risk from contaminated sites.

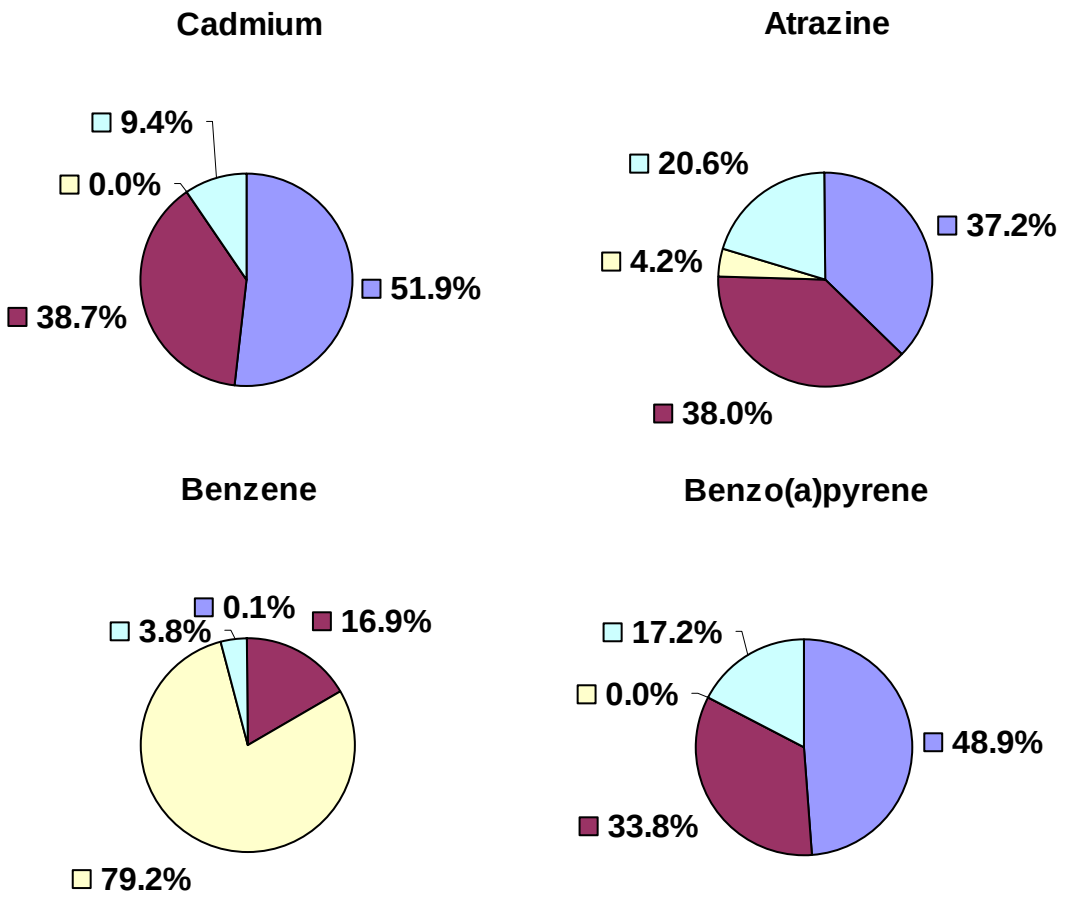


Figure 7.1 Contribution of the separate exposure pathways to total exposure of human adults to cadmium, atrazine, benzene and benzo(a)pyrene. Values are averages from seven European exposure models (%). (Source: Swartjes, 2002). soil ingestion ; crop consumption ; indoor air inhalation ; other exposure pathways

Bioavailability processes in oral contaminant exposure

Carrying out site-specific risk assessment studies to evaluate human health risks from contaminated sites requires an evaluation of the hazardous effects of soil contaminants when taken up by the human body. Toxicity values of the contaminant as such are in many cases available, but they are often based on the external dose of the chemical to test animals, rather than the actual internal dose which is generally not known. Therefore, the term “absolute bioavailability” is introduced giving a direct link between the external dose and the internal dose and indicating the fraction of an orally ingested contaminant that reaches systemic circulation. For example, ingestion of 10 mg Pb will thus result in a 50% bioavailable fraction if 5 mg is taken up in the systemic tract. However, these absolute bioavailability values are useless when considering ingestion of contaminated matrices. The bioavailable fraction of contaminants in soil is generally lower than the contaminants that are used in the toxicity for deriving toxicity values. Hence, the term “relative bioavailability” needs to be introduced which takes into account the differences between bioavailability in the soil matrix and bioavailability in the toxicity test matrix. Briefly, the relative bioavailability is the ratio between the amount of a contaminant reaching systemic circulation when ingested with soil and the same amount obtained when ingested in the toxicity experiment (Grøn and Andersen, 2004). Following from the above example: if 10 mg soil-bound Pb is ingested and the relative bioavailability is 20%, then the Pb bioavailability is: 10mg * 50% * 20% = 1mg.

The current assumption that a soil contaminant has a relative bioavailability factor of 100% compared to the toxicity study significantly impacts the outcome of a risk assessment. The limited knowledge concerning the gastrointestinal parameters which govern the releas of a soil contaminant, respectively the complexation adds to the uncertainty of the risk assessment in general. Additionally, the calculated risks and deduced soil quality guidelines may be too conservative leading to unrealistically high exposure levels which would unnecessarily raise the economical costs associated to soil remediation initiatives. Moreover, the current approach does not allow placing remediation priority on one contaminated site or another, since the calculated exposure dose is always the same (NRC, 2003). Hence, risk assessments of contaminated soils would be more accurate when the luminal processes that determine the contaminant bioavailability are clarified and better understood. This would improve the development of better *in vitro* methods that allow to study the gastrointestinal tract. These methods can then be used in site-specific risk assessment studies in which the bioavailable fraction of a toxicant is assessed under relevant conditions.

Metabolic potency of colon microbiota

Cancer of the colon is typically a disease of the economically "developed" world. In Europe, cancer of the colon and rectum is the second most common cancer for both men and women (Ferlay et al., 2000) (Table 7.1). Scientific studies suggest that diet plays a significant role in the aetiology of colorectal cancer (Gill and Rowland, 2002; WHO, 2002). The colon microbiota are thought to play a fundamental role in the general health status of a human subject, both positively e.g. by the production of short chain fatty acids (SCFA) which provide energy to colonocytes or by stimulating the host's immune system, but also negatively e.g. by the production of hazardous metabolites or colonization of pathogenic bacteria. Especially the involvement of the colon microbiota in colorectal cancer by the production of carcinogenic metabolites from dietary constituents has been intensively investigated and discussed (Ilett et al. 1990; Rumney et al. 1993; Gill and Rowland 1998; Hughes and Rowland, 2000; Hirayama et al., 2000; Aura et al. 2002; Humblot et al. 2004a). Several enzymes and metabolites have been identified that are directly or indirectly related to colorectal carcinogenesis (Ilett et al., 1990; McBain and Macfarlane, 1998; Hughes and Rowland, 2000). Yet, there are only limited data on the potency of the colon microbiota to directly bioactivate environmental xenobiotics. If the gut microbiota are able to bioactivate chemicals that are normally inactive and if the bioactivated metabolites significantly contribute to the total risk of a contaminated sample, this could have consequences for current human health risk assessment. Chemicals that are not absorbed in the small intestine but that are available to microbial biotransformation may become as yet hazardous to the colonocytes in an activated form. This hypothesis requires further investigation and is of relevance to the aetiology of colorectal cancer in general.

Table 7.1 Incidence of most common cancers in 2000. (Source: Ferlay et al., 2000)

	Males			Females		
	Rank	**Cancer**	**New cases (thousands)**	**Rank**	**Cancer**	**New cases (thousands)**
World	1	Lung	902	1	Breast	1050
	2	Stomach	558	2	Cervix	471
	3	Prostate	543	3	Colon/rectum	446
	4	Colon/rectum	499	4	Lung	337
	5	Liver	398	5	Stomach	318
Developed countries	1	Lung	471	1	Breast	579
	2	Prostate	416	2	Colon/rectum	292
	3	Colon/rectum	319	3	Lung	175
	4	Stomach	208	4	Stomach	125
	5	Bladder	164	5	Corpus uteri	114
Developing Countries	1	Lung	431	1	Breast	471
	2	Stomach	350	2	Cervix	379
	3	Liver	325	3	Stomach	193
	4	Oesophagus	224	4	Lung	162
	5	Colon/rectum	180	5	Colon/rectum	154

Research findings

The most important findings from this research can be located in two different aspects of oral exposure to soil contaminants. The different research chapters and most important findings are schematized in Figure 7.2.

I. Firstly, assessing the risks from ingested contaminated soil largely depends on the correct measurement of the **bioavailable fraction** of the compound, whereas the toxicity of the compound as such is usually well known. The contaminant release and complexation reactions that are part of the **bioaccessibility process** in the intestinal lumen play **a decisive role in the bioavailability process, prior to absorption and biotransformation**, but there is currently no consensus how to measure it.

- In Chapter 2, a unique comparison study of five *in vitro* methods of the human gastrointestinal tract was conducted to **identify the most crucial parameters that influence the bioaccessibility** of lead in a contaminated soil.
- In Chapter 3, luminal desorption and complexation processes were studied in detail for a PAH contaminated soil. The research conclusions from this chapter, together with the knowledge obtained from Chapter 2 **give the required indications of how to interpret and measure the bioaccessible fraction of an ingested contaminant.**

II. Secondly, ingested contaminants that are not transported across the intestinal wall may partly become **available to intestinal microorganisms**. The role of metabolic activities of the gut microbiota in relation to cancer has been investigated before, but more research is needed to find out to what extent these microbiota can directly transform ingested environmental contaminants.

- In Chapter 4, an **analytical method was developed to detect microbial PAH metabolites** in the complexity of an *in vitro* cultured intestinal suspension.
- In Chapter 5, it was shown for the first time that the **colon microbiota have the potency to convert PAHs to bioactive compounds**, more in particular metabolites with estrogenic properties. Such processes are as yet not considered in risk assessment practice.
- In Chapter 6, a potential **added value of chicory inulin** was explored. **Inulin exerted strong inhibitory effects towards microbial PAH bioactivation** which opens new perspectives for dealing with ingested contaminants from the environment or food.

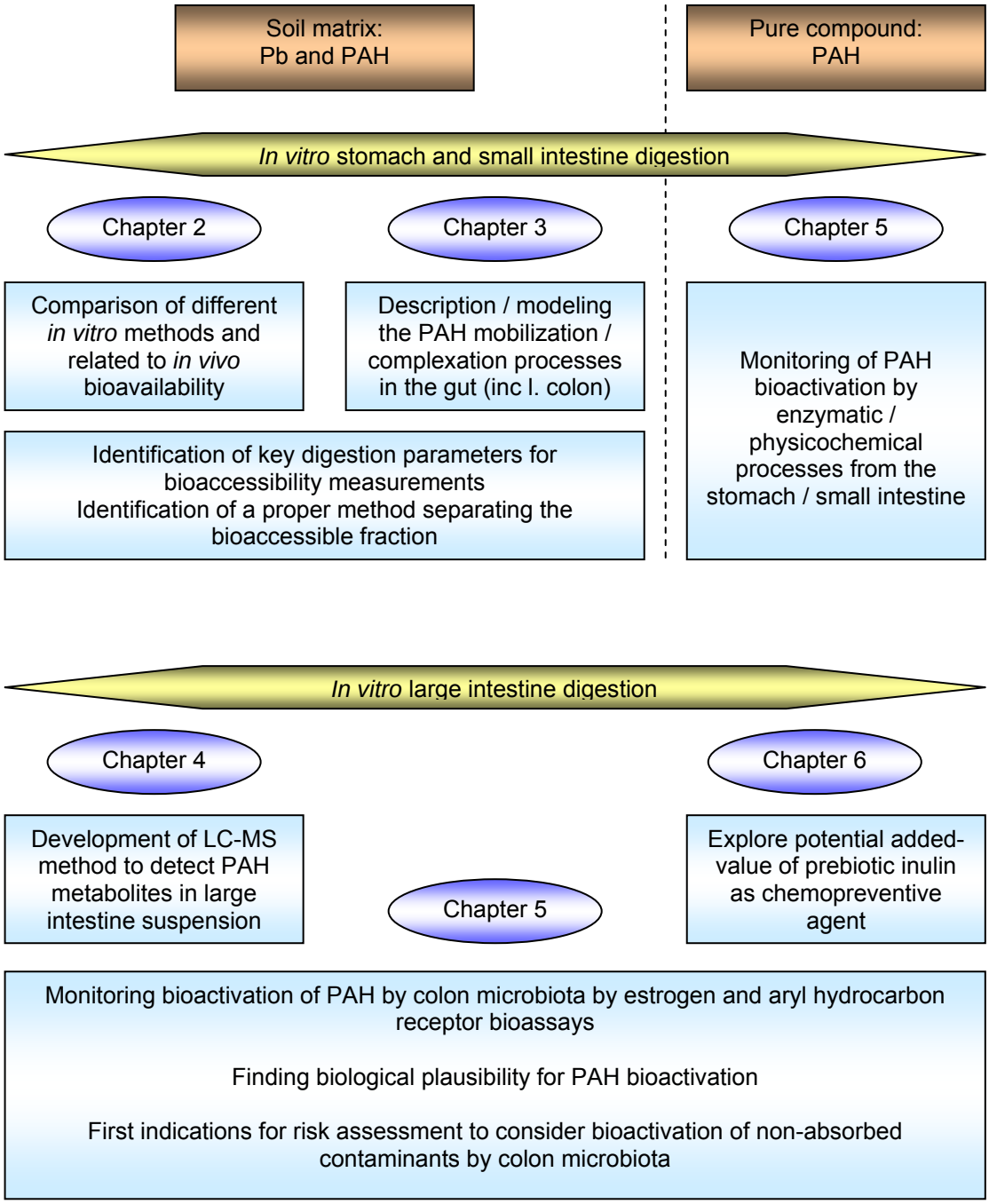


Figure 7.2 Schematic research overview for locating the different research chapters

General discussion

Bioaccessibility: reviewing research data

The first two research chapters of this dissertation, **Chapter 2** and **3** specifically dealt with luminal processes of soil-bound contaminants in the *in vitro* gastrointestinal tract and how to obtain bioaccessibility data from these digestions. **Chapter 2** describes the research conducted with BARGE (BioAccessibility Research Group Europe). Five *in vitro* models were used to determine the bioaccessible fraction of soil-borne lead under fed and fasted conditions. The models were compared against each other and it was investigated how well they relate to the oral bioavailability values of lead from the same soil, as obtained by Maddaloni et al. (1998). An important observation is that under fed conditions, all models yielded bioaccessibility data that were significantly higher than the bioavailable fraction. This shows that the generated bioaccessibility values are conservative estimators of bioavailability. The underestimation of bioavailability of the SHIME, PBET and DIN models under fasted conditions is more problematic. The fact that all models except TIM used digestion parameters for small children may be a possible explanation for this discrepancy. To illustrate, the SHIME model assumes a hypothetical child soil ingestion of 1 g d^{-1} leading to low liquid to soil ratios (L/S) of 25. This may hinder the lead mobilization from the soil matrix.

However, a more important feature to remember from **Chapter 2** was the general difference between fed and fasted bioaccessibility. Although the variability between the different models was still fairly high, almost every model showed higher bioaccessibility values under fed conditions, this in contrast to the *in vivo* bioavailability data which were lower under fed conditions (2.5% versus 26.2% under fasted conditions). The TIM model followed the latter trend and had bioaccessibility values that closely approached the *in vivo* bioavailability results. Also the RIVM method followed the same trend, but had bioaccessibility values that were much higher than the *in vivo* bioavailability values. The positive influence of food compounds towards an increase in bioaccessibility values was already observed and discussed in the previous comparison study from BARGE (Oomen et al., 2002) and it was also proposed by Hack and Selenka (1996). It is however in sharp contrast with the observations of Maddaloni et al. (1998) and other researchers who found lower contaminant bioavailability values under fed conditions (James et al., 1985; Blake et al., 1983). Maddaloni et al. (1998) explain this *in vivo* trend by the fact that nutritional components such as calcium, iron, and other cations interact strongly as competitors of Pb uptake so that Pb absorption generally increases as dietary levels of these nutrients decrease

(Muschak, 1991). However, this explanation specifically refers to the intestinal absorption process, whereas *in vitro* models only simulate luminal processes, not intestinal transport nor biotransformation. It is much more likeley that the presence of fibres and lipids plays a key role in the release of lead from its soil matrix. The difference in bioaccessibility values between models and the influence of fed conditions towards contaminant bioaccessibility needs further discussion.

Before addressing bioaccessibility further, a closer look will be taken at some of the results of **Chapter 3**, because additional clues are given here to help elucidate the luminal processes to which ingested contaminants are subjected. Here, a PAH contaminated soil was subjected to stomach, small intestine and colon conditions and the PAH release from the soil matrix was monitored. It was not the purpose of this research chapter to calculate the bioaccessible fraction, but to specifically focus on typical luminal processes such as contaminant mobilization from a matrix and complexation to other compounds in the intestinal suspension. A mathematical model was also developed to verify some of the observed trends and thus get a better understanding of these effects. Although **Chapter 3** dealt with an organic chemical, PAH, instead of a metal, Pb, in **Chapter 2**, a similar effect from food components was observed towards the bioaccessible fraction. Adding nutrition to a stomach digest of the PAH contaminated soil significantly raised the released PAH fraction with 65% compared to fasted conditions without food compounds. The explanation is that nutrition contains higher concentrations of organic matter under the form of fibres, polysaccharides, fats or other compounds. This organic matter in the gastrointestinal solution provides more complexation niches for PAHs in solution as was already indicated for other organics by Hack and Selenka (1996) and Oomen et al. (2002). If the complexation is considerable, this might affect some of the PAH compounds present in soil, by shifting the equilibrium towards desorption from the soil matrix. The signifant effects from bile salts towards PAH desorption and complexation confirm earlier observations of Oomen et al. (2000).

An important aspect to remember from **Chapter 3** is that the mathematical model aided in confirming the modulating effect of food compounds and bile towards PAH desorption from the soil matrix and the formation of novel complexes with bile. The latter phenomenon occurred with bile as such or bile in combination with organic matter (including microbial biomass when present in the colon). The mathematical model showed that there is a dual effect from organic compounds in the intestinal suspension. On the one hand, desorption of PAHs from the soil matrix may be enhanced due to the provision of complexation niches. On the other hand, complexes

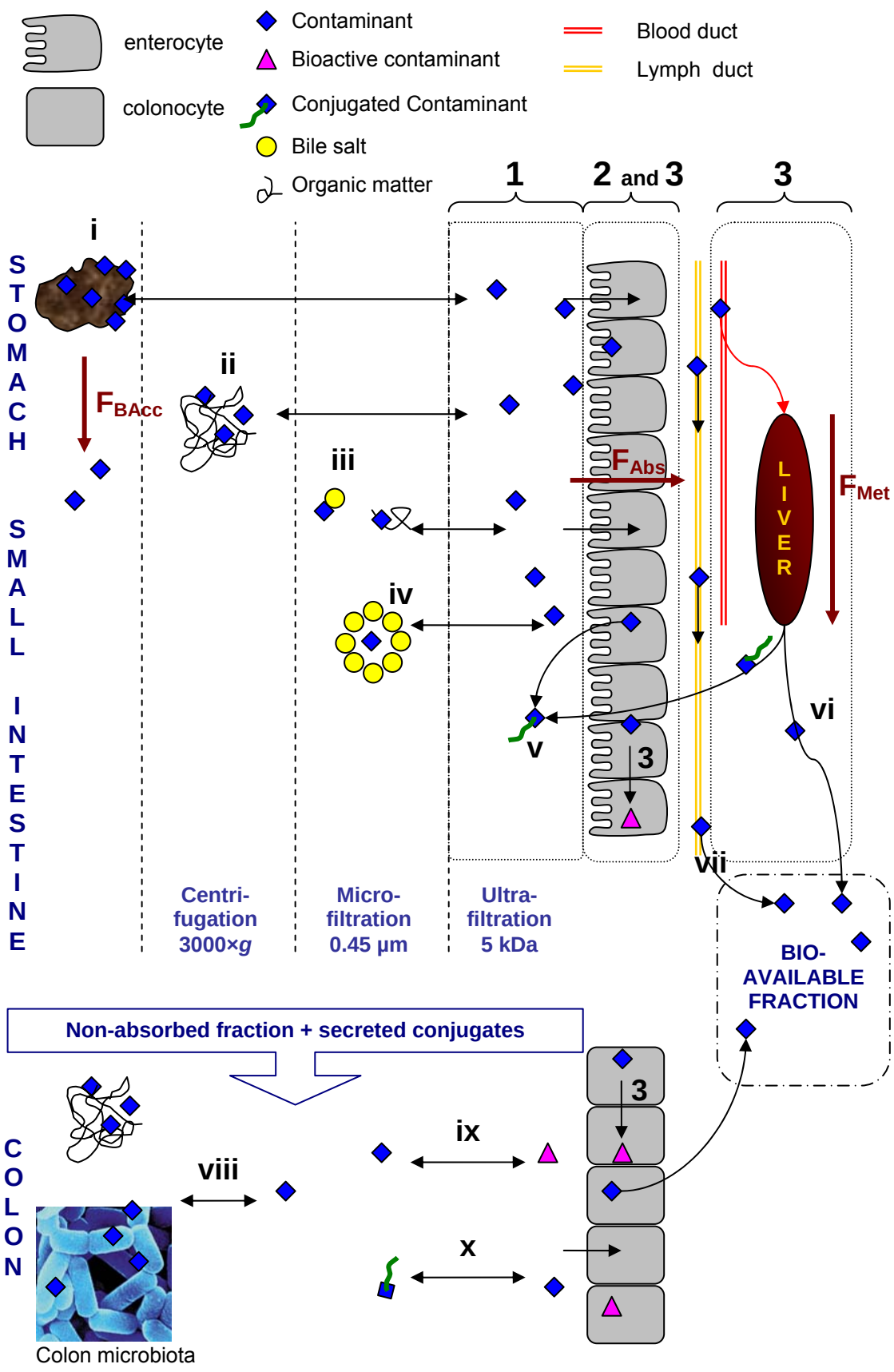
may be formed that are too large to come into consideration for getting absorbed across the intestinal epithelium. This directly affects the bioaccessible fraction of the PAH contaminant. It is important to note that the mathematical model was specifically developed using parameters from the different digest conditions and the PAH contaminated soil. It can thus not be extended to and applied for other situations or contaminated sites.

Bioaccessibility: towards a better interpretation

Bringing together the different observations and conclusions made from **Chapter 2** and **3**, we can now try to find an explanation for the differences in bioaccessibility values between the different *in vitro* models and why the TIM model obtained bioaccessibility values that were lower under fed than under fasted conditions. Soil-borne contaminants that enter the gastrointestinal tract will be subjected to various mechanical (grinding, kneeding), physico-chemical (dissolution, sorption) and enzymatic (protease, amylase…) processes. Microbial interactions are only predominant in the colon, and are not considered in this discussion of bioaccessibility. The different processes that bring an ingested contaminant from soil-bound to bioavailable are depicted in Figure 7.3.

Upon ingestion of a soil-bound contaminant (i) an equilibrium of sorption and mobilization from the soil matrix may partly dissolve the contaminant in the gastrointestinal lumen. The freely solubilized contaminant fraction may then interact with (ii) particulate organic matter, (iii) dissolved organic matter or (iv) it may be taken up in bile salt micelles. Bile salts and even lipids and certain proteins may act as a carrier vehicle for apolar and cationic contaminants towards the surface of the intestinal epithelium.

Figure 7.3 Processes towards bioavailability of ingested soil-borne contaminants:
1. bioaccessibity, 2. intestinal absorption, 3. human metabolism.
i) contaminant desorption
ii) interaction with particulate organic matter
iii) interaction with dissolved organic matter
iv) uptake in bile salt micelles
v) biotransformation to conjugated metabolites.
vi) fraction that passes the liver, vii) uptake in lymph duct.
viii) interaction with colon microbiota
ix) microbial conversion to bioactive metabolites
x) microbial deconjugation


enterocyte
colonocyte
Contaminant
Bioactive contaminant
Conjugated Contaminant
Bile salt
Organic matter
Blood duct
Lymph duct
1
2 and 3
3
STOMACH
SMALL INTESTINE
i
ii
iii
iv
v
vi
vii
viii
ix
x
F_{BAcc}
F_{Abs}
F_{Met}
LIVER
Centri-fugation 3000×g
Micro-filtration 0.45 µm
Ultra-filtration 5 kDa
BIO-AVAILABLE FRACTION
Non-absorbed fraction + secreted conjugates
COLON
Colon microbiota

At the surface of the enterocytes, a micro-acidic environment prevails which destabilizes micelles causing fatty acids to release and become available for intestinal transport (NRC, 2003). For hydrophobic contaminants specifically, co-transport with fatty acids is thus made possible. If absorption takes place, the contaminant may (v) undergo biotransformation processes and may be excreted in a conjugated form back into the intestinal lumen. Biotransformation takes place in the enterocytes and/or liver, the latter may produce conjugates for both biliary as urinary excretion. The contaminant fraction that (vi) passes the liver or that is (vii) transported to the lymph ducts upon absorption are taken up in the systemic circulation and are bioavailable. Hence, the oral bioavailability of a chemical depends on three crucial steps: 1) bioaccessibility, 2) intestinal absorption and 3) metabolism by human biotransformation enzymes and is calculated as:

$$F = F_{BAcc} * F_{Abs} * F_{Met} \qquad (1)$$

with F the bioavailable fraction, F_{BAcc} being the bioaccessible fraction, F_{Abs} being the fraction absorbed and F_{Met} being the fraction which escapes human metabolism. To accurately predict oral bioavailability, it should be assessed what the sensitivity is of these 3 factors to matrix effects. Clearly, the metabolism factor F_{Met} is insensitive to luminal matrix effects, whereas the bioaccessible fraction F_{BAcc} is highly sensitive since matrix effects determine the luminal processes of mobilization, complexation, desorption… From the above description of Figure 7.3, the intestinal absorption F_{Abs} can not be influenced by matrix effects since chemicals must be present in a free form at the surface of the intestinal epithelium to be considered for absorption. This interpretation contrasts the previous assumption that the intestinal absorption process is sensitive to matrix effects such as bile salts or organic matter (Hack & Selenka, 1996; Reynolds et al., 1998; Weber and Lanno; 2001; Grøn and Andersen, 2003). Only the chemical form and chemical properties of the contaminant will determine whether it is absorbed or not. Absorption is thus clearly separated from the luminal matrix effects which are part of the bioaccessibility process. (*note: there are some exceptions with regards to pinocytosis where particulates may be absorbed. This transport process is however less common*).

This discussion immediately falls back to the definition of bioaccessibility: **bioaccessibility describes the fraction of the chemical that desorbs from the soil matrix and is available for intestinal absorption** (Ruby et al., 1999). Although this definition is generally agreed upon, it is remarkable to see that there are so many approaches to separate the bioaccessible fraction from the non-bioaccessible fraction:

3000×*g*, 3500×*g* or 7000×*g* centrifugation, 0.45µm filtration, 5 kDa ultrafiltration... (Ruby et al., 1996; Hack and Selenka, 1996; Oomen et al. 2002; Waisberg et al., 2004; Arkbåge et al. 2004). As inferred from the definition, a bioaccessible contaminant is available for intestinal absorption. Hence, it should be present as a free compound at the surface of the intestinal wall and released from micelles or other complexes. This means that a stringent method is needed to separate this fraction. The currently applied separation methods of centrifugation, microfiltration, 5 kDa ultrafiltration pertain to the different sizes of the carrier-contaminant complex that will be left in the supernatant or filtrate (Figure 7.3). The distinction between the different complexes by different separation methods in this figure should not be literally interpreted, but merely gives an indication of the different fractions that exist. To show that the separation method is indeed very crucial, the RIVM institute recently performed experiments in which the bioaccessibility of lead from the Bunker Hill soil was measured under fed conditions with the same breakfast formulation as the one used in the *in vivo* study from Maddaloni et al. (1998) and that used with the TIM model (**Chapter 2**). Different separation methods were compared to one another and had a significant influence on the outcome of the tests (Table 7.2).

Table 7.2 Differences in bioaccessibility of lead in Bunker Hill soil under fed conditions depending on the separation method

Separation method	Bioaccessibility (%)
Centrifugation (3000×*g*)	31.5
Microfiltration (0.45 µm)	22
Ultrafiltration (5 kDa)	3.5

From these data and the previous discussion, it can be concluded that the **separation method is a crucial parameter to consider when interpreting bioaccessibility results**. This also has consequences when comparing lead bioaccessibility to oral bioavailability data using equation 1. For lead as a heavy metal, liver metabolism is not relevant, so a F_{Met} value of 100% is assumed. The intestinal transport of lead is inherent to the intestinal epithelium and is a constant, hence F_{Abs}=constant. Since the separation method is not part of the digestion process, the bioaccessible fraction F_{Bacc} should also be a constant under well-defined circumstances. Assuming that ultrafiltration is the closest approach to bioaccessibility, F_{BAcc} is 3.5% and 7% for the RIVM and TIM method, respectively. Remaining differences between the 2 models can be explained by the different digestion characteristics. The other (milder) separation methods generate values that

overestimate the actual bioaccessible fraction in various degrees, necessitating the use of larger correction factors to calculate oral bioavailability. This adds to the uncertainty of the risk assessment process. However, it should be kept in mind that using more stringent separation methods such as ultrafiltration will also decrease the level of conservacy, which is something that needs careful consideration, especially for human health risk assessment where safety factors must always be included.

In summary, the different sizes and types of the carrier-contaminant complex that are related to different separation methods cause the fundamental difference in the current bioaccessibility measurements of chemicals. Besides that, there are still methodological differences due to other digestion characteristics between *in vitro* methods of the gastrointestinal tract. The different bioaccessibility data from **Chapter 2** can partially be explained by the difference in the digestion characteristics such as L/S ratios, stomach pH, bile content and presence of food compounds (Hack and Selenka, 1996; Hamel et al., 1998). Further research from BARGE will focus on both digestion characteristics as the separation methods, which will allow to give additional recommendations to researcher and risk assessers that want to develop a protocol to test the bioaccessible fraction of ingested soil contaminants. This will allow to give a conservative estimate of the orally bioavailable fraction.

Colon microbiota and ingested contaminants

From Figure 7.3, it can be inferred that a certain amount of ingested chemicals will reach the colon region of the human GI tract. This fraction incorporates compounds that are not transported across the intestinal epithelium, compounds that are excreted by the P-glycoprotein efflux metabolism from the enterocytes and compounds that are secreted as conjugated metabolites from biotransformation processes in the enterocytes or hepatocytes. Besides this, the colon suspension, or colon chyme, also contains undigested food components, plant chemicals, microbial fermentation products, microbial metabolites and secreted compounds from the intestinal epithelium. Several of these colon chyme constituents may present a health risk by exposing the colorectal mucosa with mutagenic compounds in the chyme. To illustrate, fecal mutagenicity studies have indicated that environmental rather than genetic factors determine the risk of colorectal cancer in a population (reviewed by De Kok and van Maanen, 2000). In addition, intestinal microorganisms are significantly associated with cancer development both in a negative way by the production of toxic

components, as in a positive way by the suppression of hazardous microbial groups and the inhibition of (pro-)carcinogen production (reviewed by Hughes and Rowland, 2000). Within the context of this doctoral research, the importance of studying colon processes, both physico-chemical as microbiological, is illustrated by the interaction of the colon matrix and colon microbiota with PAHs that reach the colon region.

As toxic effects towards the intestinal epithelium are most probably attributed to soluble reactive substances in the intestinal suspension, the aqueous phase of human faeces (fecal water) is used to assess the toxicity and genotoxicity of harmful substances in the colon chyme (Gill and Rowland, 2002). In this respect, it is important to better understand how contaminants in the colon suspension interact with substances from the colon matrix. **Chapter 3** showed how PAHs in the colon may adsorb to microbial biomass, besides the soil matrix, residual food compounds and other intestinal components (Figure 7.3 (viii)). These additional complexation sites in the colon suspension will affect the fraction of contaminants that is bioaccessible. **Chapter 3** only addressed some of the complexation processes in the colon suspension. Further investigations should therefore focus on actual bioaccessibility measurements as outlined in the previous discussion. Such research may be particularly interesting when studying the absorption of persistent organic pollutants (POP) by the colon wall. Although the lower surface area of the colon epithelium and its increased tightness generally lowers colon absorption compared to small intestine absorption, it seems that some hydrophobic compounds, such as POPs, are better absorbed in the colon region of the GI tract (Ungell et al., 1998). This is important since the contact of hazardous compounds with the colon epithelium and subseqent absorption may give detrimental health effects. In general, not much research has been performed to study the bioavailability of ingested environmental contaminants in the colon region. Yet, some results are available from the pharmaceutical sector. Certain drugs are unstable under the low pH of the stomach or high enzymatical or bile content of the small intestine. Since this sensitivity (partly) decreases their activity, there is much interest to specifically deliver drugs to the colon where absorption can take place under less stringent conditions (Yokoe et al., 2003; Nykanen et al., 2001). The principle of the so called slow-release systems for colon-targeted drug delivery are often microbially triggered (Sinha and Kumria, 2003) and allow the intestinal absorption of hydrophobic compounds in the colon region of the human GI tract.

Where **Chapter 3** targeted the complexation of contaminants in the colon region and the process towards bioaccessibility, **Chapters 4** and **5** addressed the presence of bioactive contaminants in colon suspension. The current focus of risk assessment for the oral exposure to environmental contaminants lies on the human bioactivation processes by cytochrome P450 complexes in enterocytes and hepatocytes. There are however many indications that the intestinal microbiota can inactivate or bioactivate a wide variety of chemical agents from diet, biliary excretion or microbial metabolites (McBain and Macfarlane, 1998). Since it is very much conceivable that the intestinal microbiota may purport similar effects towards ingested contaminants, the metabolic potency of colon microbiota to convert PAHs to bioactive transformation products was assessed (Figure 7.3 (ix)). In **Chapter 4**, an analytical methodology using LC-MS was developed to detect microbial hydroxy-PAH metabolites in the complex colon suspension. For these experiments, it was important to consider the interference of the chyme matrix background with the analysis of hydroxy-PAHs which may suffer from poor limits of detection or low reproducibility. Future research efforts to analyze microbial biotransformation products, biliary excreted metabolites or food chemicals in the chyme should therefore spend enough time to optimize the chemical analysis method and get rid of the background interference. The detection of 1-OH pyrene and 7-OH benzo(a)pyrene upon incubation of parent PAHs with colon microbiota reveals that intestinal microorganisms indeed have a metabolic potency towards organic chemicals.

Moreover, the bioassay experiments conducted in **Chapter 5** showed that the colon microbiota are able to convert PAHs towards compounds that show an affinity for the human estrogen receptor. Although a positive response in the estrogen receptor bioassay does not necessarily predict endogenous transcription (Gozgit et al., 2004), these microbial bioactivation phenomena may not be neglected. Microbial bioactivation is not covered in current risk assessment practice, but it has already been extensively discussed when reviewing the relationships between diet and cancer and the role of intestinal microorganisms (Ilett et al., 1990; McBaine and Macfarlane, 1998; Hughes and Rowland, 2000; Gill and Rowland, 2002). The type of microbial metabolic processes are highly diverse and usually reductive (Ilett et al., 1990). Yet, the hydroxylation of PAHs as discussed in **Chapter 4** and **5** is not an isolated case of oxidative reactions. The hydroxylation of IQ to 7-OH IQ by intestinal *Eubacterium* and *Clostridium* species have been reported several times (Van Tassel et al., 1990; Rumney et al., 1993; Kassie et al., 2001) and advanced analytical techniques such as Nuclear Magnetic Resonance (NMR) are being used for the identification and isolation process of microbial metabolites in the complex matrix of the intestinal suspension

(Humblot et al. 2004b; Rabot, 2004). The conclusions from **Chapter 5** show that for oral exposure to environmental contaminants, further research should focus on the microbial bioactivation potency in the intestinal lumen. It should also be assessed to what extent microbially bioactivated contaminants may contribute to the total risk that environmental contaminants pose towards human health.

From Figure 7.3 (x), it should also be kept in mind that there is an indirect bioactivation route of intestinal microorganisms towards contaminants. Conjugated metabolites of contaminants can enter the colon lumen as a result of biotransformation processes in the enterocytes or in the hepatocytes, followed by secretion into the intestinal lumen. These more hydrophilic conjugates are normally not available for intestinal absorption allowing them to be removed from the body with the feces. However, the colon microbiota possess several enzymes that may deconjugate the phase II metabolites, regenerating the more hydrophobic parent compound or phase I metabolite which is then again more available to absorption (Gill and Rowland, 2002). In this way for instance, bacterial β-glucuronidase activities can hydrolyze glucuronidated conjugates and may thus delay the excretion of harmful exogenous compounds (Weisburger, 1971). This was also demonstrated by Takada et al. (1982) who observed a reduced number of azoxymethane induced tumours in the rat colon upon administration of β-glucuronidase inhibitors.

The microbial bioactivation of ingested PAHs in **Chapters 4** and **5** and the indirect bioactivation through microbial deconjugation enzymes, indicate the important role of the colon microbiota in the generation of toxic compounds. Since such bacterial enzymatic activities are often diet related, it would be interesting to modulate the bioactivation potency through dietary factors. Several studies have shown that the diet strongly modulates the metabolic activity from intestinal microbiota (Hambly et al., 1997) and changes in the microbial community composition have been observed to influence the metabolism of DNA-reactive carcinogens (Chadwick et al., 1992). It has been demonstrated that oligosaccharides such as FOS, GOS, or inulin inhibited the formation of heterocyclic aromatic amines (HCA) (Shin et al., 2003), while probiotic bacteria as dietary supplements exerted detoxifying properties towards hazardous chemicals in the GI tract (Rowland et al., 1998).

This formed the starting point for the research described in **Chapter 6**, in which the use of native chicory inulin as inhibitory compound for PAH bioactivation was explored. The results from the estrogen bioassay showed that inulin administration significantly decreased the formation of estrogenic compounds in PAH incubated colon samples, specifically in the ascending colon and to a lesser extent also the transverse colon. The most important effect from inulin administration towards the microbial community was the significant increase in Bifidobacteria, as indicated by plate counts, realtime PCR and DGGE. Inulin-type fructans are composed of β-D-fructofuranoses attached by β-2→1 linkages (Roberfoid, 1999) that can be broken by Bifidobacteria specifically, thus giving a nutritional advantage over other microbial groups in the gut (Roberfoid, 2001). This may suppress microbial groups that are for instance responsible for the production of bioactive PAH metabolites, other carcinogens or toxins. This prebiotic effect was accompanied by a significant increase in short chain fatty acid production, primarily butyrate and propionate and a decrease in ammonia production which can be considered as general indicators of a lower incidence of cancer (Jan et al., 2002; Scheppach et al., 1995). Additionally, the hazardous β-glucuronidase production was significantly decreased by inulin addition which would increase *in vivo* the excretion of PAH conjugates with the fecal material.

These observations indicate that the prebiotic effect of inulin addition acts on several aspects which may all be related to toxic PAH metabolites in the colon. Future research should further elucidate which organisms are probably suppressed and it may be investigated whether these are also responsible for the production of hazardous PAH metabolites. Additionally, other dietary factors and the influence of human age and/or physiology towards contaminant bioactivation in general needs to be further addressed.

In summary of this general discussion, some take home messages can be formulated.

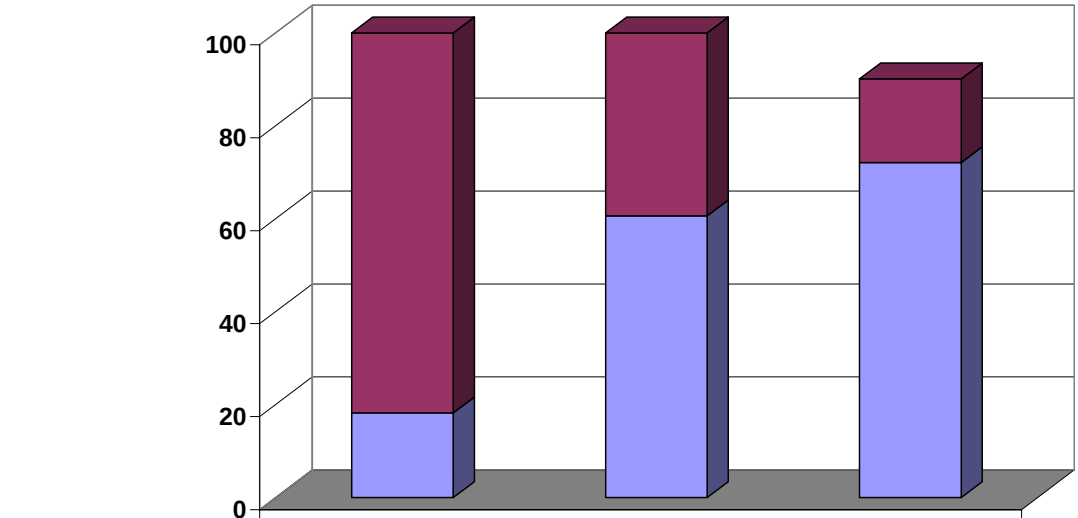
1. *In vitro* methods of the human GI tract allow **mechanistic** studies and **high-throughput** screenings of many environmental samples in a cost-efficient way. This makes **bioaccessibility** measurements an important tool when performing site-specific risk evaluations.
2. **Bioaccessbility** measurements always generate **conservative** estimators of the oral bioavailabiltiy, provided that the bioaccessible fraction is accurately measured.
3. The **separation method** is one of the most crucial parameters to consider when interpreting bioaccessibility data and when identifying correction factors that relate bioaccessibility to oral bioavailability. **Ultrafiltration** seems to give the closest estimate of the actual bioaccessible fraction, but it should be kept in mind that the level of conservacy is lower with more stringent separation methods.
4. **Human colon microbiota** are able to **directly convert PAHs into estrogenic metabolites**. This direct bioactivation of PAHs by colon microbiota has not been observed before and further research should investigate what microbial groups are responsible for the conversion and to what extent bioactivated PAHs contribute to the total health risk from ingested PAHs.
5. **If significant**, microbial conversion of ingested contaminants should be considered in **risk assessment**.
6. **Prebiotic inulin** has an **added-value** by its **chemopreventive** properties towards the bioactivation of PAHs. Inulin elicits strong bifidogenic effects and further research should focus on which microorganisms are suppressed by inulin supplementation and if these are related to the observed conversion of PAHs to estrogenic metabolites.

Research perspectives

From the framework of this research – oral exposure to environmental contaminants – several future research topics can be identified that are directly or indirectly related to human health risk assessment (HHRA). These can be situated in the increasing importance and relevance of rapid and adequate screening tests for the site-specific risk evaluation of an increasing number of contaminated areas and the risk evaluation from new and existing chemicals that are brought on the market and reach the environmental compartments. Additionally, the influence of intestinal microorganisms on the bioactivation of ingested compounds, both environmental contaminants as dietary constituents, is a field of research which is largely unexplored.

Soil contaminants

The world economy has experienced an exponential increase since the Industrial Revolution and in the last couple of decades it has been constantly increasing with 30 billion € every year, putting it at roughly 43.75 trillion € in 2002 (Nationmaster, 2004). It is remarkable that the developed countries, which represent only 18% of the world's population, are responsible for 60% of the world gross domestic product (GDP) (Figure 7.4).



	% world population	% world GDP	ecological footprint (km2 per 1000 inhabitants)
Developing countries	81.8	39.4	18.0
Developed countries	18.3	60.6	72.1

Figure 7.4 Comparison of the global GDP and ecological footprint between developed and developing countries.

The ecological footprint from the developed countries is a factor 4 larger than that from the developing countries and in numerous “developed” regions, the environmental buffer capacitiy to adsorb and convert contaminants has been exceeded for several decades already. Disturbances in the ecosystem are therefore no longer small and quickly restored, but lead to major environmental threats coming from the excessive use of the environment as a sink for our pollution and waste streams, thus exceeding the environmental capacities to adsorb and convert them. This same capacity for contaminant adsorption and conversion can also be allocated to soils.

Soils are recipients for contaminants from combustion processes, sludge and pesticide applications, leakage from industrial sites, landfilling and other waste disposal methods (EEA, 1999). In the Europe of the 12 alone, more than 1,500,000 sites are potentially contaminated (EEA, 1999) (Table 7.3). The majority of these sites are located in regions which are heavily industrialized or densely populated. For example, in Flanders it is estimated that around 4% of the total surface area is potentially contaminated. Moreover, it is assumed that much more areas that are not yet registered are also contaminated, since in most countries detailed surveys of soil conditions and site-specific risk assessment studies are still in process. The remediation costs for those sites that are currently known to have contaminant concentrations above the intervention values is estimated to be more than 120 billion €, which brings a financial cost of 300 € per European inhabitant for the next generation.

Table 7.3 Available data on the number of potentially contaminated sites, for selected categories and countries (n.i.: no information). (EEA, 1999)

	Contaminated sites	
	Identified (screening complete)	Estimated total
Austria	28000	80000
Belgium	5528	22500
Denmark	37000	40000
Finland	10396	25 000
France	n.i.	800 000
Germany	202 880	240 000
Italy	8 873	n.i.
Netherlands	n.i.	120 000
Sweden	7 000	n.i.
Switzerland	35000	50000
United Kingdom	n.i.	100000

The afore mentioned numbers just include soil contamination from local sources. However, diffuse sources also significantly contribute to soil pollution by atmospheric deposition of contaminated particles in urban areas (from traffic and industry), farming practices (fertilizers and antibiotics), irrigation (wastewater reuse) and pesticide treatment. In fact, Western Europe's anticipated growth in road transport and the integration of additional industrial activities in the expanded Europe of 25 countries is expected for the next 10 years to increase emission levels of several chemicals such as cadmium, mercury, PAH and pesticides, whereas PCB, PCDD, PCDF and lead emissions will decrease (Figure 7.4). For example, pesticide application is a major diffusive emission source since in many cases less than 5% of the pesticides actually reach the soil surface, depending on the weather conditions (Lennartz et al., 1995) and the rest is dissipated into the environment. Several reports show that the atmospheric deposition of persistent organic pollutants such as PCDF, PCB and PAH is gradually increasing over the last century reaching levels for PAHs of 0.07 kg ha^{-2} yr^{-1} (Sanders et al., 1992; Sanders et al., 1993; Green et al., 2001) which is 1 to 2 orders of magnitude lower than the pesticide application in agriculture (1-4 kg ha^{-2} yr^{-1}). Yet, due to their persistence in the environment, this has lead to PAH levels that range between 0.7 and 40.7 mg kg^{-1} in urban soils (Mielke et al., 2001) and between 8 and 336 mg kg^{-1} in road dust (Menzie et al., 1992).

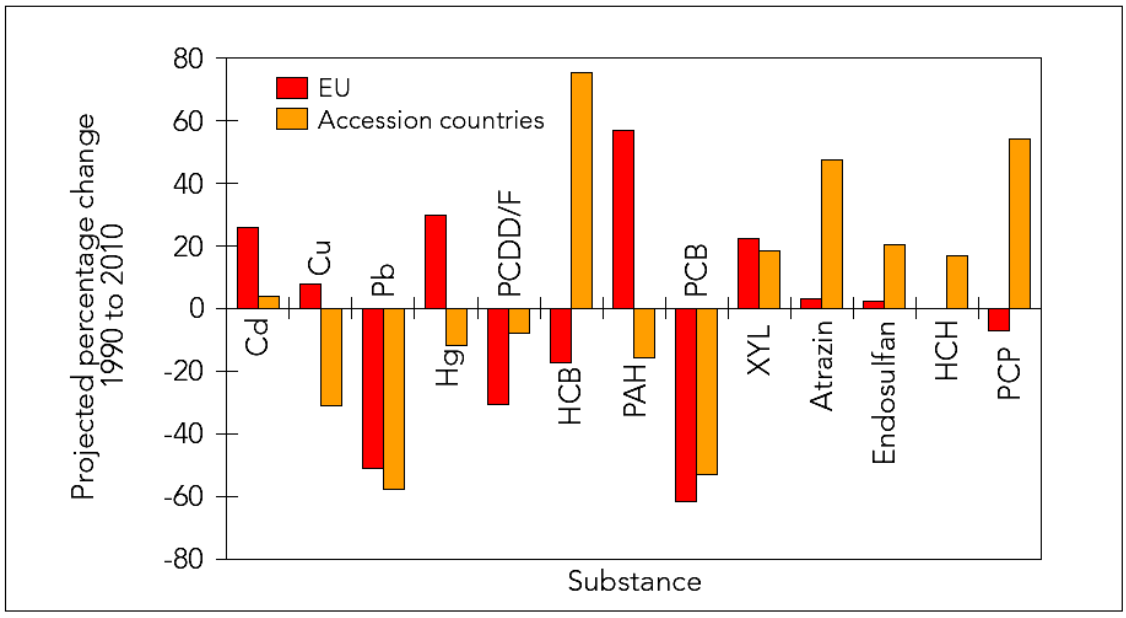


Figure 7.4 Projected percentage changes 1990 to 2010 in emissions of selected chemicals (EEA, 1999)

Additionally, investigations on the top layer of an alleged non-contaminated site in the Black Forest, Germany, revealed PAH soil concentrations between 100 and 500 μg kg^{-1} and a leachable PAH fraction of around 0.5 μg L^{-1} which is higher than the European drinking water limit for PAHs (0.2 μg L^{-1}) (Halm and Gratwohl, 2003). It can be inferred from these numbers that in the near future, more and more people may possibly be exposed to contaminated soils, both in densely populated as more rural areas. The importance of human health risk assessment as site-specific assessments will therefore only increase. Hence, reliable and accurate high-throughput methods, which are also less expensive and less time-consuming than *in vivo* methods, are needed to allow an efficient, fast and accurate site-specific risk assessment.

REACH: registration, evaluation and authorisation of chemicals

The release of manufactured chemical products in the environment by anthropogenic activities is ever increasing since 1993 (CEFIC, 2002). Currently, there are 20,000 to 70,000 chemical compounds on the European market, many of them being hazardous for the environment in general or human health in particular. Several of these compounds are not yet completely characterized wih regards to their environmental fate and their (eco)toxicity, hence the actual environmental or human health risk that they represent is not well known (Figure 7.5).

Within this scope, the European Union is currently preparing new directives for setting up REACH – Registration, Evaluation Authorisation and Restriction of Chemicals – a new evaluation system which forces manufacturers and importers of chemical compounds to register their products. The endgoal is to better assess the impact of chemicals towards public health and the environment. The REACH approach thus places a large responsibility with the industry to perform risk assessment studies and to comply with the safety standards that are put forward by the EU. Nearly 30,000 chemical compounds should be documented by the year 2016. To realize the goals of the REACH legislation it is of the utmost importance, both practically and scientifically, to prioritize which chemicals need to be handled first. The European Chemical Industry (CEFIC) and European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) have therefore chosen for a targeted risk assessment approach, improved data acquisition, *in silico* and *in vitro* methods to evaluate chemicals that will be brought onto the market. In parallel, environmental and (eco)toxicological scientists and environmental regulators are preparing new recommendations for the OECD in setting up new guidelines that make possible a more efficient screening procedure of

new chemicals with respect to their Persistent, Bioaccumulative and Toxic properties (PBT) (STEP workshop, 2004). It is within this framework that an enormous amount of work needs to be undertaken for assessing human health risks from chemicals. With regards to human oral exposure, the use of *in vitro* models would fit in the CEFIC strategy of a 'targeted risk assessment' which allows faster screening and evaluation. Of course, manufactured chemicals are not the only compounds that are hazardous. The environment is constantly exposed to a multiplicity of natural compounds and compounds that have entered the different environmental compartments by anthropogenic activities.

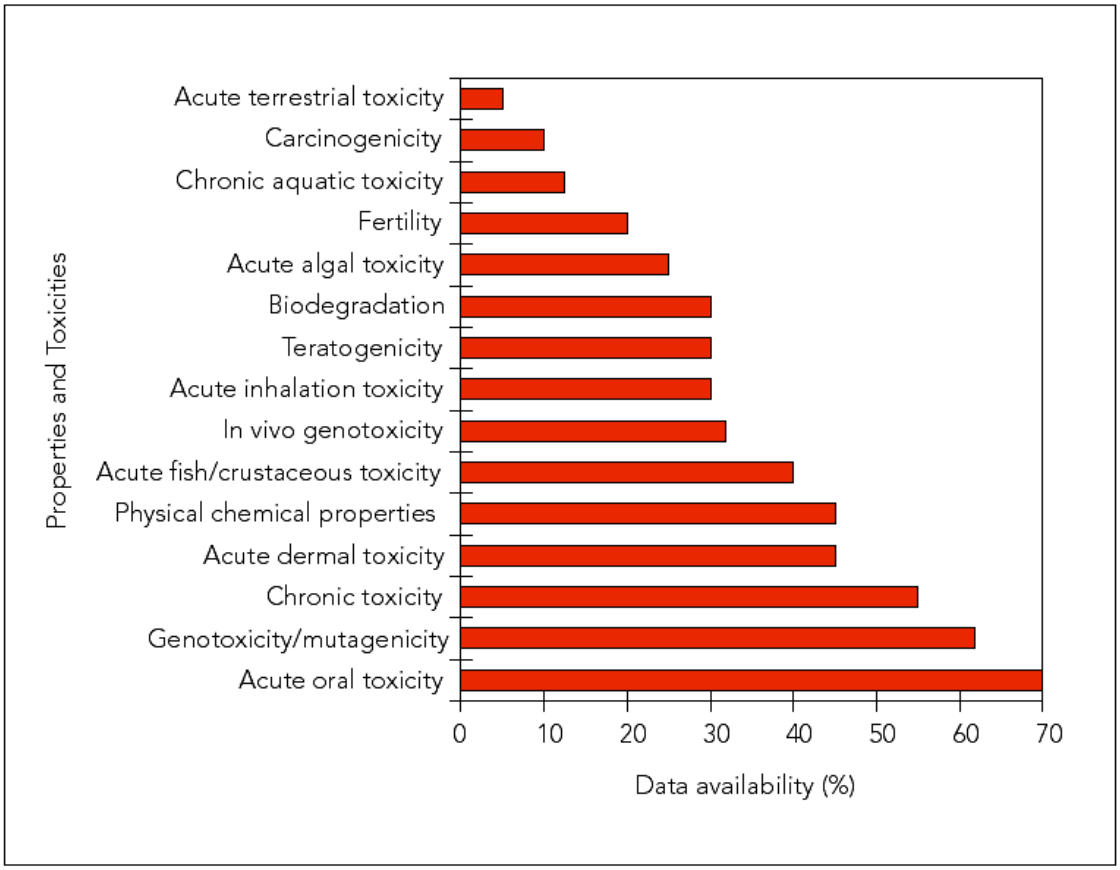


Figure 7.5 Availability of data on 2472 high-production volume chemicals submitted to the European Chemicals Bureau (EEA, 1999)

Food contaminants

Food consumption represents an important pathway for human exposure to chemicals from a variety of sources (Dougherty et al., 2000). Not only are there chemicals of concern in the natural product (phytoestrogens, plant or animal toxins…), there are also hazardous chemicals that are produced during food processing such as cooking, broiling or grilling. For instance, the consumption of broiled red meat shows a

strong correlation with colon and rectum tumours in particular due to the production of potential carcinogens are PAHs and heterocyclic aromatic amines (HCA) during food processing (Norat et al., 2002; Murtaugh et al., 2004). Chemicals are also added to food for specific technological functions such as food preservation, flavouring or colouring. Additionally, chemicals from normal agricultural practice such as pesticides or veterinary drugs may be present as residuals in human nutrition. External contamination of food by environmental pollutants such as dioxins, PCBs and PAHs may be important sources of human exposure. For example, diet is the main source of human background exposure to POPs such as PCDD, PCB and HCB (hexachlorobenzene) (Schlummer et al. 1998). The best-known example of food contamination is probably the presence of PCBs and dioxins in food and livestock feedstuffs in Belgium in 1999 and 2000. Given the widespread occurrence or production of contaminants in human nutrition and the associated serious health risks, prevention of further food contamination must be a national health policy priority in every country (Schafer and Kegley, 2002) and efficient procedures should be in place to estimate the health risk of food contaminants, taking into account all sources of exposure (WHO 2004). Such assessments are based on global toxicological evaluations performed by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and on estimates of exposure to chemicals in the diet.

As with environmental contaminants, much scientific attention has been payed to the metabolization of ingested food contaminants by human biotransformation enzymes, whereas many researchers have now pointed out that colon microbiota also significantly contribute to the hazardous health effects, for example by the conversion of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) to the mutagenic 7-keto derivative (7-OHIQ) (Van Tassel et al. 1990; Rumney et al. 1993; Kassie et al. 2001). The intestinal microorganisms are generally believed to be strongly involved in the aetiology of colorectal cancer not only by the direct conversion of chemicals but also by the deconjugation of excreted phase II metabolites in the intestinal lumen (Gill and Rowland, 2002). Therefore, the problem of food contaminants and the role of intestinal microorganisms in the associated health risks opens several research perspectives 1) to further clarify microbial-ecological mechanisms that influence the conversion of food chemicals to hazardous metabolites, 2) to identify dietary characteristics that modulate these processes and 3) to investigate whether dietary supplements such as prebiotics or probiotics may serve as chemopreventive agents as already indicated by Rowland et al. (1998).

Integrated approach for studying intestinal processes

To summarize, the growing awareness of the relationship between general food safety, environmental contaminants and human health points out the significance of conducting research in the field of human nutrition, gastrointestinal microbiology and health relevant biotransformation processes. The recently published global strategy on diet, physicial activity and health (WHO, 2004), together with the founding of the European Food Safety Authority (EFSA) and the innovation center of the Flemish food industry (Flanders' Food) provides an extra stimulus for the scientific world, internationally and nationally, to address human health issues related to general food and environmental safety. The demand for demonstrating the safety of a broad group of environmental and food chemicals will require a reliable means of assessing priorities. It is inconceivable to achieve this by using in-depth toxicological tests and costly and time-consuming *in vivo* methods. Rather, prioritization is much needed and should be made possible using high-throughput screening techniques that are still reliable with regards to exposure assessment (bioavailability processes) and toxicity assessment (biotransformation and toxicity processes).

This framework provides several research perspectives for the use of *in vitro* models such as the SHIME or other complex models (Minekus et al. 1995) to study the GI tract. These studies are quicker and more reproducible than *in vivo* methods and for particular mechanisms, they can be more accurate. Metabolization reactions from cultured intestinal microbiota can be studied in a mechanistic way and in much more detail. Moreover, the dynamics and metabolism of microbial populations of interest in the intestinal lumen can be adequately monitored using a combination of physicochemical, microbiological and enzymatical analyses. Further developments in the application of non-radiolabelled technology such as nuclear magnetic resonance (NMR) spectroscopy, will presumably increase the understanding of toxicokinetics and thus improve the process of hazard characterisation (Dybing et al., 2002). In addition, molecular fingerprint and quantification techniques such as PCR-DGGE, Fluorescent In Situ Hybridization (FISH), realtime PCR and Flow Cytometry now make it possible to quantify and characterize an *in vitro* cultured microbial community in great detail (Langendijk et al., 1995; Rigottier-Gois et al., 2003). Moreover, combining *in vitro* digestion technology such as the SHIME with the culture of monolayers of enterocytes or colonocytes such as Caco-2 and the culture of mucus secreting HT29 cells opens up an additional field of research. It would allow to investigate intestinal transport processes (Stein et al., 2000; Oomen et al., 2001), immunological response (Hooper et al,. 2001; Cummings et al., 2004), bacterial adhesion to the intestinal epithelium

(Drudy et al., 2001; Zarate et al., 2002), and the effects of specific compounds of interest at the genetic level of cultured intestinal cells (Granato et al., 2004). Additionally, fecal water genotoxicity measurements are possible by Single Cell Gel Electrophoresis techniques and are currently investigated with the Comet Assay (Venturi et al., 1997; Burns and Rowland, 2004).

Of course, much attention needs to be payed to the validation of the outcome of *in vitro* studies with *in vivo* animal studies. Yet, the combination of *in vitro* gastrointestinal digestion technology with cell cultures for measuring host-specific processes allows to carry out mechanistic investigations. These can be used to elucidate for example the way how intestinal microorganisms interact with the host by producing metabolites, adhering to the intestinal epithelium, stimulating immune response and modulating carcinogenic effects from dietary or environmental constituents. This will also allow for a better interpretation and extrapolation of results to *in vivo* situations. In this way, a combination of novel *in vitro* techniques with *in vivo* experiments will provide a better understanding of underlying mechanisms in the aetiology of adverse health effects from environmental and food contaminants.

SUMMARY

Ingestion is a predominant exposure route for environmental contaminants to enter the human body. Common exposure scenarios are the ingestion of contaminated soil by small children due to their typical hand-mouth behavior and the direct or indirect ingestion of soil and dust particulates by construction workers in contaminated areas. Additionally, food consumption is an important exposure pathway by the presence of contaminated soil particles on badly cleaned vegetables, the presence of contaminants inside the plants due to plant uptake, bioaccumulation of hazardous organic chemicals throughout the foodchain or by the production of hazardous compounds during food processing. To correctly assess the risks that these ingested chemicals pose towards human health, a basic understanding is required of the processes that determine how available an ingested chemical is for absorption by the body and the processes that determine its possible bioactivation and toxicity. These two aspects of oral uptake of environmental contaminants were highlighted in this doctoral research and further investigated.

In the first part, processes of bioavailability were investigated. The bioavailability process determines what fraction of an ingested contaminant reaches the blood compartment. For this, the chemical compound needs desorption from its matrix in the gastrointestinal lumen, transport across the intestinal epithelium and passage through the liver without being biotransformed and excreted from the body. Although pharmacokinetic models and *in vivo* toxicity studies have generated many useful data to elucidate the processes of intestinal absorption and human biotransformation, the desorption and complexation processes in the gut lumen are often a black box to risk assessers. Therefore, *in vitro* models of the human gastrointestinal tract have been developed which simulate the physicochemical, enzymatic and microbial processes occurring in the gut lumen. Studying these luminal processes allow to measure a contaminant's bioaccessibility, which is basically the fraction of the chemical that releases from its matrix and becomes available for intestinal absorption. As the bioaccessibility process is an important prerequisite to bioavailability, these *in vitro* methods generate a conservative estimator for the bioavailable contaminant fraction, which is important to maintain safety factors in risk assessment. Additionally, these methods allow a high-throughput, flexible and reproducible screening of many samples, which is interesting when conducting site-specific risk assessment studies.

Comparison of bioaccessibility data from a lead contaminated soil as generated from five European *in vitro* methods (PBET, DIN, RIVM, SHIME and TIM) of the human gastrointestinal tract revealed that a large variability exists between the current models, a confirmation of earlier observations where other soil-borne contaminants were investigated. Many of the differences could be (partly) explained by the different digestion parameters that were applied in a specific *in vitro* method, such as stomach pH, digestion time, the amount of added digestive suspension, what risk group was simulated (child, adult)… However, the presence of food compounds had a more significant impact on the outcome of the bioaccessibility measurements. Several *in vitro* methods displayed higher bioaccessibility data when fed conditions were simulated, whereas the reverse trend was observed from an *in vivo* study where the presence of food compounds lowered the lead bioavailability. Clearly, this brings about uncertainty when interpreting the outcome from *in vitro* bioaccessibility measurements. A first clue towards the explanation for this fundamental difference was given by the observation that the TIM model, which simulates the human gut in much detail, also generated lower bioaccessibility data under fed conditions and that it closely approached the *in vivo* bioavailability of lead. Although the higher complexity of the model may explain the more accurate estimates of bioavailability, the most important difference of the TIM model with the other *in vitro* methods is the use of ultrafiltration membranes to separate the bioaccessible lead fraction from the intestinal suspension. This is a much more stringent method than centrifugation at 3000×*g* or 0.45 µm filtration which are used by the other methods. Ultrafiltration gets rid of larger lead-complexes which are not available for intestinal absorption. From these data it was hypothesized that the presence of food compounds may enhance the release of lead from the soil, but that complexation to these food compounds limit the lead bioaccessibility.

To study these release and complexation phenomena in more detail, batch experiments according to the principle of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) were conducted on a PAH (polycyclic aromatic hydrocarbon) contaminated soil. Although the type of chemical was different, more clues were given for elucidating some luminal processes to which ingested contaminants are subjected. As expected, specific digestion parameters such as stomach pH and liquid to solid ratio influenced the release of PAHs from the soil matrix. More importantly, the presence of dissolved and particulate organic matter had a dual impact on the soil-borne PAHs. On the one hand, the presence of organic matter in the intestinal suspension provides for additional complexation niches, hence increasing PAH desorption from the soil. On the other hand, PAH-organic matter

complexes limit the fraction of PAHs which is freely available and thus the bioaccessibility. These observations were confirmed by data from a mathematical model which was developed using standard analogue compounds for PAHs, bile salts and organic matter.

In summary, it can be concluded that *in vitro* methods of the human gut can be used for bioaccessibility measurements only when they reflect as closely as possible the digestive parameters of the risk group on a specific contaminated site. Therefore, no standard *in vitro* method exists, but a standard protocol should be developed for risk assessers which parameters need to be considered when specific digestion experiments need to be performed. This would greatly enhance the predictive power of bioaccessibility measurements to the oral bioavailability of ingested contaminants.

In a second part of this doctoral research, the interaction of ingested contaminants with *in vitro* cultured colon microbiota was evaluated using the SHIME method. The reason for this is that current risk assessment studies on ingested contaminants primarily consider toxification and/or detoxification processes that take place by human biotransformation enzymes in the intestine enterocytes or liver hepatocytes. However, non-absorbed contaminants or secreted chemical conjugates in the intestinal lumen may be transported to the colon region and become available to the vast microbial community that resides in the human colon. Thus far, it was not known to what extent intestinal microbiota may be able to convert hazardous organic compounds to metabolites that are harmless or worse, that possess more toxic properties than the parent compound. To study this microbial biotransformation potency, PAHs were chosen as model compound, both in pure form as present in a soil matrix.

Firstly, a LC-MS (liquid chromatography mass spectrometry) method was developed and optimized to detect PAH metabolites in colon suspension that was sampled from the SHIME and incubated with pure PAH parent compounds. The focus was put on hydroxy-PAHs since preliminary experiments with bioassays indicated that estrogenic metabolites may be obtained. Several hydroxy-PAH metabolites are known for their estrogenic properties. Although the complex nature of the colon suspension matrix represented some problems with background interfernce, decent limits of detection at ppb level were obtained. Interestingly, analysis of pyrene and benzo(a)pyrene incubated colon samples revealed the presence of 1-hydroxypyrene and 7-hydroxy benzo(a)pyrene, which is the first analytical evidence that colon microbiota are able to convert PAHs without the presence or interference from human biotransformation enzymes.

Secondly, it was assessed whether these colon suspensions elicited a bioactive response in a couple of bioassays. It is known that PAHs as such are not estrogenically active. Use of a yeast-based estrogen receptor bioassay revealed that colon microbiota were indeed able to convert PAHs to metabolites with estrogenic properties. Zero-response data from stomach and small intestine incubated samples showed that this microbial bioactivation potency was colon-specific. Moreover, the observed estrogenicity was not merely a matrix effect, since inactivation of the colon microbiota also took away the estrogenicity of incubated samples. More importantly, analysis of PAH contaminated soil indicated that this bioactivation also occurs at environmentally relevant concentrations. Further research should focus to what extent the bioactive PAHmetabolites contribute to the total risk from ingested PAHs, but clearly, absorption of bioactive PAH metabolites may present hazards to both the colon and rectum and thus present a health risk that should be taken into consideration.

Finally, it was investigated whether addition of native chicory inulin could inhibit the extent of microbial PAH bioactivation reactions. Inulin is generally agreed to exert prebiotic effects as it stimulates health-promoting bacteria in the human gut such as Bifidobacteria or Lactobacilli. However, it is also hypothesized that it may exert chemopreventive effects by the indirect suppression of microbial groups such as certain pathogens that may be responsible for the hazardous conversion of ingested chemicals, such as PAHs. Supplementation of inulin during several weeks to a full-scale SHIME reactor had significant inhibitory effects towards PAH bioactivation to estrogenic metabolites, especially in the proximal colon compartments. Interestingly, the strongest prebiotic effects were also observed in this region of the colon, indicating an indirect relationship with the chemopreventive effects from inulin. This added-value aspect of inulin as prebiotic and probably other prebiotics needs further elucidation, for example by the identification of the microbial groups that are responsible for the PAH bioactivation and those microbial groups that are suppressed during inulin addtion.

SAMENVATTING

Ingestie is een belangrijke blootstellingroute waarlangs milieucontaminanten het menselijke lichaam binnentreden. Veel voorkomende blootstellingwegen zijn de ingestie van gecontamineerde bodem door kinderen via hun typisch hand-mond gedrag en de directe of indirecte ingestie van bodem- en stofdeeltjes door arbeiders in vervuilde gebieden. Daarbij vormt voedselconsumptie eveneens een belangrijke blootstellingweg door de aanwezigheid van vervuilde bodemdeeltjes op slecht gereinigde groenten, de opname van contaminanten door de plant zelf, bioaccumulatie van organische contaminanten doorheen de voedselketen en de productie van risico-houdende componenten tijdens de voedselbereiding. Het juist inschatten van de risico's die oraal opgenomen componenten inhouden voor de menselijke gezondheid, vergt een basiskennis over deze processen die bepalen hoe beschikbaar een stof is voor absorptie door het lichaam en de processen die een eventuele bioactivatie en toxiciteit veroorzaken. Deze twee aspecten van orale opname van milieucontaminanten werden in dit doctoraal onderzoek toegelicht en verder onderzocht.

In een eerste deel werden processen van biobeschikbaarheid bestudeerd. Biobeschikbaarheid bepaalt welke fractie van een ingeslikte contaminant de bloedbaan bereikt. Hiervoor moet de component eerst vrijgesteld worden van zijn matrix in het maag-darm kanaal, getransporteerd worden doorheen het intestinaal epithelium en de lever passeren zonder te worden gebiotransformeerd wat kan leiden tot excretie uit het lichaam. Niettegenstaande farmacokinetische modellen en *in vivo* toxiciteitstudies zeer bruikbare informatie opleveren voor het ophelderen van processen van intestinale absorptie en menselijke biotransformatie, zijn de desorptie- en complexatieprocessen in het darmlumen momenteel niet goed gekend bij risico-evaluatie. Daarom werden *in vitro* methodes ontwikkeld die de physicochemische, enzymatische en microbiële processen van het menselijke spijsverteringskanaal nabootsen. Het bestuderen van deze luminale processen laat toe om de "biotoegankelijkheid" van een contaminant te meten, wat in principe de fractie is die in het lumen vrijgesteld wordt van de matrix en die beschikbaar is voor intestinaal transport. Aangezien biotoegankelijkheid een belangrijke voorwaarde vormt voor biobeschikbaarheid, leveren deze *in vitro* methodes een inschatting op van de biobeschikbare fractie met een veiligheidsmarge, wat belangrijk is voor risico-evaluatie. Daarenboven kunnen met deze methodes vrij veel

stalen op een snelle, flexibele en reproduceerbare wijze gescreend worden, wat interessant is voor plaats-specificieke risico-evaluatiestudies.

Vergelijking van de biotoegankelijkheidswaarden van een lood-gecontamineerde bodem die bekomen werden met vijf Europese *in vitro* methodes (PBET, DIN, RIVM, SHIME en TIM) van het menselijke spijsverteringskanaal, toonden aan dat er een grote variabiliteit bestond tussen de verschillende modellen. Dit is een bevestiging van vorige studies waarbij andere bodemcontaminanten werden bestudeerd. Veel van de verschillen konden (gedeeltelijk) verklaard worden door de verschillende digestieparameters die werden toegepast voor een specifieke *in vitro* methode, zoals de maag pH, digestietijd, de hoeveelheid verteringsuspensie of de risicogroep (kind, volwassene) die wordt bestudeerd. De aanwezigheid van voedingscomponenten bleek echter een meer significante impact te hebben op de biotoegankelijkheid. Verschillende *in vitro* methodes gaven hogere waarden aan indien gevoede omstandigheden werden gesimuleerd, dit in tegenstelling tot een *in vivo* studie op dezelfde bodem waar de gevoede omstandigheden leidden tot lagere biobeschikbaarheidswaarden. Deze bevindingen brengen onzekerheid teweeg bij de interpretatie van *in vitro* resultaten. Een eerste indicatie om dit fundamentele verschil te verklaren werd geleverd door het TIM model dat het maag-darm kanaal vrij gedetailleerd nabootst. Hierbij werd vastgesteld dat gevoede omstandigheden resulteerden in een lagere biotoegankelijkheid, net zoals de *in vivo* biobeschikbaarheid. Niettegenstaande de hogere graad van complexiteit een verklaring kan zijn voor de meer accurate inschattingen van het TIM model, is het belangrijkste verschil met de andere modellen de methode die gebruikt worden om de biotoegankelijke fractie af te scheiden, nl. ultrafiltratie. Dit is een veel strengere methode dan centrifugatie bij 3000×*g* of 0.45 µm filtratie die bij de andere modellen worden gebruikt. Ultrafiltratie houdt geen rekening met complexen van lood met organisch materiaal die niet beschikbaar zijn voor intestinaal transport. Uit deze resultaten werd besloten dat de aanwezigheid van voedingscomponenten de vrijstelling van lood uit de bodem kan verhogen, maar dat complexatie met deze voedseldeeltjes de biotoegankelijkheid van lood limiteren.

Om deze vrijstelling- en complexatieprocessen in meer detail te bestuderen werden batchexperimenten volgens het principe van de Simulator van het Humaan Intestinaal Microbieel Ecosysteem (SHIME) uitgevoerd op een PAK (polycyclisch aromatische koolwaterstoffen) gecontamineerde bodem van een voormalig recreatiegebied. Zoals verwacht waren specifieke digestieparameters zoals de maag pH van invloed op de PAK vrijstelling uit de bodemmatrix. Heel belangrijk was de waarneming dat opgelost en particulair organisch materiaal een tweeledig effect had

op de PAKs. Aan de ene kant, vormt organisch materiaal in de intestinale suspensie een bron van complexatieniches, waardoor PAK desorptie in de hand wordt gewerkt. Langs de andere kant beperken complexen van PAKs met organisch materiaal de fractie die vrij in oplossing is en dus ook de biotoegankelijke fractie. Dit werd bevestigd door een wiskundig model dat ontwikkeld werd met behulp van standaardcomponenten voor PAKs, galzouten en organisch materiaal.

Samenvattend kan worden besloten dat *in vitro* methoden voor het menselijke maag-darm kanaal enkel gebruikt kunnen worden voor biotoegankelijkheidsmetingen indien de digestieparameters zo nauwkeurig mogelijk deze van de risico-groep benaderen bij de beoordeling van een gecontamineerde site. Er is dus geen standaardmethode voorhanden. Wel kan een standaardprotocol worden opgesteld waar risico-inschatters zich aan kunnen houden om te weten welke parameters en welke types van digestie-experimenten moeten worden uitgevoerd. Dit zal de voorspellende kracht voor biobeschikbaarheidsmetingen voor ingeslikte contaminanten sterk verhogen.

In een tweede deel van dit doctoraal onderzoek, werd de interactie van oraal opgenomen contaminanten met *in vitro* gecultiveerde colon microbiota bestudeerd via de SHIME methode. Huidige risico-evaluatiestudies houden momenteel voornamelijk rekening met toxificatie en/of detoxificatieprocessen die plaatsvinden in de enterocyten of hepatocyten door menselijke biotransformatie enzymes. Niet-geabsorbeerde contaminanten of gesecreteerde conjugaten ervan in het darmlumen kunnen echter getransporteerd worden naar de colon en beschikbaar komen voor de enorme microbiële gemeenschap die in de colon verblijft. Tot hier toe was het niet bekend in welke mate intestinale microbiota in staat zijn om schadelijke organische componenten om te vormen tot metabolieten die in het ergste geval toxischer zijn dan de moedercomponent. Om deze microbiële biotransformatie capaciteit te onderzoeken, werden PAKs als modelcomponenten gebruikt, zowel in pure vorm als in een bodemmatrix.

Eerst en vooral werd een LC-MS (liquid chromatography mass spectrometry) methode ontwikkeld en geoptimalizeerd om PAK metabolieten te detecteren in colonsuspensie van de SHIME reactor die werd geïncubeerd met zuivere PAKs. De focus werd gelegd op gehydroxyleerde metabolieten, vermits preliminaire experimenten aantoonden dat mogelijk estrogene componenten werden verkregen. Verschillende hydroxy-PAK metabolieten zijn bekend voor hun estrogene eigenschappen. De complexiteit van de colonmatrix leverde aanvankelijk problemen met achtergrondinterferentie, maar toch werden detectielimieten op ppb-niveau

bereikt. Opmerkelijk was dat pyreen en benzo(a)pyreen geïncubeerde colonstalen aanleiding gaven tot de detectie van 1-hydroxypyreen en 7-hydroxy benzo(a)pyreen, wat meteen het eerste analytische bewijs vormde dat colon microbiota in staat zijn om PAKs om te vormen zonder dat daarvoor menselijke biotransformatie enzymes nodig waren.

Vervolgens werd er nagegaan of PAK geïncubeerde colon suspensies een bioactieve respons gaven in een aantal bioassays. PAKs op zich zijn niet estrogeen actief, maar gebuik van de de estrogene receptor bioassay toonde aan dat colonmicrobiota inderdaad in staat waren om PAKs om te vormen tot metabolieten met estrogene eigenschappen. Deze omzetting was colonspecifiek aangezien geïncubeerde maag- en dunne darmstalen geen positieve respons gaven in de estrogene test. De waargenomen estrogeniciteit was ook niet afkomstig van matrix effecten, aangezien inactivatie van de colonmicrobiota geen aanleiding gaf tot stalen met estrogene eigenschappen. Meer nog, analyse van de PAK gecontamineerde bodem toonde aan dat deze bioactivatie ook optreedt bij milieu-relevante concentraties. Verder onderzoek moet uitwijzen in welke mate deze bioactieve PAK metabolieten bijdragen tot het totale risico van de oraal opgenomen PAKs. Het is echter duidelijk dat absorptie van bioactieve PAKs in het colon of rectum een risico vormt naar de gezondheid toe.

Tenslotte werd onderzocht of toediening van inuline de graad van microbiële PAK biotransformaties kon verminderen. Inuline wordt algemeen aangenomen prebiotische effecten uit te oefenen door het stimuleren van gezondheidsbevorderende bacteriën, zoals Bifidobacteria of Lactobacilli, in de darm. Er wordt echter ook gesteld dat het een chemopreventieve werking heeft door de indirecte onderdrukking van microbiële groepen, bv. pathogenen, die verantwoordelijk zijn voor de schadelijke omzettingen van PAKs. Toevoegen van inuline gedurende een aantal weken aan de SHIME reactor had een significant inhiberend effect naar de PAK bioactivatie tot estrogene componenten toe, vooral in de proximale coloncompartimenten van de SHIME. Een interessante vaststelling was dat de sterkste prebiotische effecten ook in deze regio van de colon werden waargenomen, wat wijst op een indirect verband met de chemopreventieve effecten van inuline. Deze toegevoegde waarde van inuline als prebioticum en tevens van andere prebiotische producten moeten verder bestudeerd worden. Verder onderzoek kan uitwijzen welke microbiële groepen worden onderdrukt door inuline en of deze eveneens verantwoordelijk zijn voor schadelijke omzettingsprocessen in het colon.

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EDUCATION

1989-1995 Latin Mathematics, Koninklijk Atheneum Zottegem (Belgium).

1995-2000 Engineer in Environmental Technology, Faculty of Bioscience Engineering, Ghent University. Thesis: “Optimalization of sludge sedimentation by moniotring and combined supplements” Supervisor: Prof. Dr. ir. W. Verstraete.

2000-2004 Doctoral education at the Faculty of Biosciences Engineering, Ghent University. Research theme: “Bioavailability of ingested environmental contaminants and interacton with the gastrointestinal microbiota.”

PROFESSIONAL ACTIVITIES

2000-2004 Academic Assisting Staff, Ghent University. Department of Biochemical and Microbial Technology, Laboratory of Microbial Ecology and Technology (LabMET).

2000-2004 PhD researcher in Applied Biological Sciences. Thesis: “Oral exposure to environmental contaminants: processes of bioavailability and interaction with intestinal microorganisms.”

2000-2004 Coordinator and collaborator of research projects in aerobic wastewater treatment, functional foods and environmental risk assessment studies, commissioned by *Avecom, Orafti, Montreal McGill University, Diamond Mills, Procter & Gamble, Cosucra, Rötgers VfT, KIBOW Biotech, AUSTEP*.

2000-2004 Coordinator and collaborator of the BARGE consortium (BioAccessibility Research Group Europe) which studies oral bioavailability processes of environmental contaminants, an initiative within the European Clarinet – Contaminated Land Rehabilitation Network.

2000-2004 Tutor of 6 students Bio-engineer during their Masters thesis en 2 students laboratory technicians during their training.

2000-2004 Responsible for computer exercises to students Bio-engineer and students from the Centre of Environmental Sanitation (Ghent University) in the courses:

- Microbial Ecological Processes
- Microbiological Research of Environmental Contamination
- Environmental Technology: Biotechnological Processes
- Advanced Environmental Biotechnology

2000-2004 Responsible for the lessons “In vitro methods for studying the human gastrointestinal tract” in the course: “Nutritional Education” (Prof. Van Camp) to students Bio-engineer

april-may 2002 Department of Biomedical Engineering, McGill University, Montreal, Canada. Installation of a SHIME-reactor (Simulator of the Human Intestinal Microbial Ecosystem).

sept-oct 2002 Collaborator at the National Water Research Institute, Saskatoon, Saskatchewan, Canada. Topic: “Screening of microbial PAH biotransformation products using LC-MS.”

nov 2002 Instructor at the 23rd SETAC congres in Salt Lake City (UT, VS). Topic: “Chemical, Molecular and Biomimetic tools to assess bioavailability”

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CONFERENCES, WORKSHOPS, SEMINARS…

Active participation

- Department for Biomedical Engineering, McGill University, Montreal, QB, Canada. Seminar: "Use of *in vitro* methods to simulate the human gastrointestinal tract for ecotoxicological studies". April 2002.
- SETAC Europe 12th Annual Meeting. Challenges in environmental risk assessment and modelling: linking basic and applied research. Vienna, Austria. Two posters. May 2002.
- Soil Science Department, Saskatoon, University of Saskatchewan, SK, Canada. Seminar: "Oral exposure to soil bound contaminants". September 2002.
- Toxicology Department, Saskatoon, University of Saskatchewan, SK, Canada. Seminar: "Oral exposure to soil bound contaminants". Oktober 2002.
- SETAC North-America 23rd Annual Meeting. Achieving Global Environmental Quality: Integrating Science & Management. Salt Lake City, UT, USA. Lecture, poster and instructor in collaboration with Steven Siciliano, Nicholas Basta, Anthony Hay. November 2002.
- Instrurama. Brussels, Belgium. Lecture: "On-line respiration measurements in wastewater treatment plants (WTP)". April 2003.
- ConSoil 8th International FZK/TNO Conference on Contaminated Soil. Gent, Belgium. Lecture and 2 Posters. April 2003
- 9th PhD symposium. Lecture. Leuven, Belgium. September 2003.

- 19th International Conference on Soils, Sediments and Water, Amherst, MA, USA. Lecture. October 2003.
- Department for Civil and Environmental Engineering, University of Illinois, Champaign-Urbana, IL, USA. Seminar: “Microbial Populations in Environmental Technology”. Oktober 2003.
- European Symposium on Environmental Biotechnology. Ostend, Belgium. Poster. April 2004.
- International Symposium on Gut Microbiology. INRA, Clermont-Ferrand, France. Lecture. June 2004.

Passive participation

- 14th Forum for Applied Biotechnology (FAB). Gent, Belgium. September 2000.
- Functional Foods: Prebiotics, probiotics and Molecular Techniques. Heverlee, Belgium. 2001.
- VLAREA EN VLAREBO: Practical information for the new waste and soil directives in Flanders. Steenokkerzeel, Belgium. Februari, 2001.
- 15th Forum for Applied Biotechnology (FAB). Gent, Belgium. September 2001.
- 50th Post Universitary Education. The impact of the Bioengineer in the biomedical sector. Gent, Belgium. December 2001.
- 16th Forum for Applied Biotechnology (FAB). Brugge, Belgium. September 2002.
- 17th Forum for Applied Biotechnology (FAB). Gent, Belgium. September 2003.
- KVIV Environmental Technology: knowledge and technology. Antwerp, Belgium. May 2004.
- Forum for Applied Biotechnology: day for SMEs. Technological evolution en cooperation opportunities in food, feed, environment en industrial biotechnology. Kortrijk. September 2004.
- STEP Workshop. Simulation Testing of Environmental Persistence. Rotterdam. Oktober 2004.

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