Monitoring Contaminants in Food Chain and their Impact on Human Health

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ABSTRACT

In recent years a great attention has been focused in Europe on the importance of food safety and the relation between diet and health. Moreover, worldwide changes in population lifestyle, together with modifications in food processing, production and distribution contributed to the eating habits of Western populations and to their reaction to recent public health emergencies. As an example, the real or alleged dioxin contamination that affected several industrialized countries has increased the interest of Authorities, producers and consumers on topics such as food safety and risks for human health deriving from contaminated food. The annual report of the European Commission Rapid Alert System for Food and Feed (RASFF) summarizes notifications on food contaminations occurred in different countries. Data analyses provided a useful tool to develop future efficient programs for food control. In this context, a working group of the PIAS project studied how specific classes of environmental contaminants (e.g, pesticides, metals, dioxins) may affect human health through the food chain. Their results are presented in this Chapter. A special section has been dedicated to highlight issues of major interest in this field, such as the determination of heavy metals and dioxins in food matrixes and biological samples; experimental models to assess the harmful effect of contaminants on human reproduction; the role of cytochrome P450 in xenobiotics metabolism. The last section of this Chapter proposes a research programme aimed at integrating aspects already faced in current literature as independent issues, but rarely considered in a holistic approach. The competences needed to pursue this goal are covered by the Italian National Research Council or by the involvement of other Italian or international institutions. The final proposal targets the youth and intends to determine the cause-effect relationship between the presence of contaminants in the diet, their accumulation in humans and the risk of chronic diseases. Key issues, such as bioavailability and adaptive response (hormesis), will be explored using suitable experimental models to suggest a functional link, at molecular level, between the onset of specific diseases and the concentrations of contaminants measured in food.

1. Background

1.1 Food safety: focusing on chemical contaminations

The introduction of genetically modified food and food incidents in Europe raised the public interest in food safety. An integrated approach to face this problem requires a strong cooperation by the food industry, food distributors, the scientific community, governments, managers and local administrators in order to build consumers' trust and confidence. The food safety certification is achieved assessing the potentially health adverse effects of food contamination. Three main food contamination groups can be identified: i. physical; ii. microbiological; iii. chemical. Physical contaminations are due to the presence of extraneous bodies in food (plastic, woods, glass and others) as the results, for example, of food packaging and/or transformation and/or storage. The substances present in those materials are not for human consumption, but

when in contact with food they migrate into it and risk of being ingested (for example, the perfluorinated chemicals used in greaseproof packaging for fast foods). Microbiological contamination refers to the presence of one or more natural biological agents, such as various bacteria, yeasts, mould, fungi, protozoa or their toxins and by-products, which can adulterate food properties and safety. Microbiological agents are responsible for "food diseases" such as food borne infections and intoxications (Botulinum, Listeria, Hepatitis A) and epidemic episodes (e.g, Salmonella enteritidis). Chemical contaminants or xenobiotics can originate from many different sources and include heavy metals, pesticides, phytopharmaceuticals, antibiotics, additives, dioxins and PCBs. Nowadays, chemical contaminants are a major concern for food safety because of the increased role of man-made chemicals due to our modern lifestyles. In fact, despite the fact that the large use of chemicals improved the quality of our lives, many of them have been reported to produce an adverse impact on human populations, animals and plants continuously exposed to a cocktail of potentially hazardous chemicals (2-4).

However, in humans and animals, diet is predominant route of many dangerous chemicals. Food is a crucial link in the chain of events starting from chemical manufacturing and ending with their presence in human blood, tissues and organs. (5). The worldwide observation of such contaminants in food shows the global scale of chemical contamination.

To assess the impact of such substances on food safety, the following question must be answered: Can the quantity and bioavailabilty of an unwanted chemical in food provide a real risk to human health? In the past, just the presence of a hazardous chemicals, whatever their concentration or weight, was considered as unsafe and adverse to health. The presence of a chemical depends on the sensitivity of the instruments used to assess it. Analysis with an increased sensitivity and different techniques showed that some chemicals, previously stated as not present, were instead only undetected.

This implies the detection itself is not necessarily representing a risk: a new approach is needed to provide a risk-based evaluation of the potential exposure, hazard, and toxicity of chemicals detected at a low-level (6).

For this reason, a threshold is needed for non toxic chemical compounds. In past decades, scientists developed different models to address this issue, concluding that the potential human health risk posed by a chemical substance depends on its inherent toxicity and exposure, including route, dose and duration. In the case of substances found in food, at least two elements must be considered. The first is their concentration level in various foods, assessed through chemical analysis. The second element is the consumed quantity of contaminated food. Bioavailability must be also considered as the capability of a dietary chemical to be absorbed and metabolized. Bioavailability is commonly assessed by measuring the amount of the ingested chemical that gets into the systemic circulation, since in most cases the specific targets and the time required to determine an effect on health are unknown. Thus, although bioavailability is critical in assessing the potential benefits or risks of a compound, it can only be studied on a comparative basis (see section 5).

It is clear that great effort is devoted to improve risk assessment and to develop common methods to be used to guarantee food quality and to protect consumers'

health. Such assessments are often based on very limited scientific information and a complete and exhaustive data set to be used to provide definitive conclusions on chemical concerns in food does not exist at the moment. In this context, results from innovative research programmes are the main sources of information to establish a common food policy and widen the existing data set.

1.2 Contamination chain: the food link

Environmental contaminants are substances present in the environment where food is grown, harvested, transported, stored, packaged, processed, and consumed. This "food chain route" of contamination implies the presence of different food contamination levels (Fig. 1), an important element to be evaluated in food safety. For example, after they are released into the environment (soil, air, water) chemical contaminants can enter plants and animals at the bottom of the food chain, to be then consumed by animals, going up in the same chain. The chemicals contained in animals and plants can enter human bodies through the diet. This concept is even more important for persistent chemicals (biomagnification) and accumulated chemicals (bioaccumulation) (e.g, pesticides, dioxins or heavy metals). These compounds are called *Persistent Organic Pollutants (POPs*) and include all those substances not rapidly degraded that keep their harmful capability towards both the environment and human health.

These substances have the following properties: i. resistance to degradation; ii. Long time persistence into the environment; iii. toxicity for humans, animals and plants; iv. accumulation in living organisms.

Bioaccumulation implies that the compound is lipid soluble and, in the absence of an adequate metabolic pathway able to eliminate it from the organism, tends to accumulate in the trophic chain. For example, polychlorinated biphenyls (PCB) are very stable organic compounds; they are highly persistent and present in air, soil and water; they are lipid soluble and bioaccumulate in animal fat, in meat and in the liver, and are transferred into milk and eggs. More than 90% of human exposure to PCBs derives from food of animal origin (7).

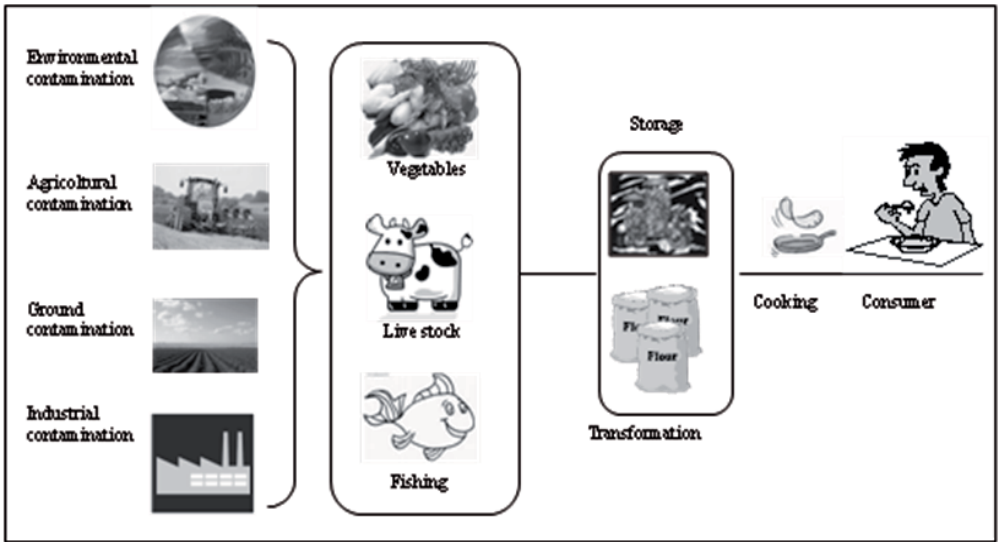

Figure 1. "The Food Chain Route"

Biomagnification is the process by which a compound increases its concentration along the food chain. Heavy metals can be bioconcentrated along the trophic chain. These substances can be involuntarily ingested with food and drinks and, once absorbed, they are distributed in tissues and organs, persisting for years or decades in some storage sites such as liver, bones and kidneys. Inorganic mercury, for example, can be converted by water micro organisms into the organic methyl mercury compound, which is then biomagnificated in higher links of the food chain. Fish, especially tuna or swordfish, can concentrate methyl mercury at high levels (8).

1.3 Chemical contamination and human health

A major concern about food contaminants is their possible adverse effects on human health. Reports on human illnesses caused by food toxic contaminants began several centuries B.C, and since then numerous episodes of food diseases have been continuously reported (9).

In recent years, many of these chemicals present in food have been detected in the blood, tissues and organs of children and adults. *POPs* are responsible for nervous systems syndromes, disruption of infant brain development, immune-pathologies, reproductive system abnormalities, cardiovascular diseases, cancers, diabetes and obesity, and some of them can act as endocrine disrupting agents. The effect on human health can be classified according to: i. acute exposure (early effects); ii. chronic exposure (long term); iii. foetal and infants exposure. Acute exposure implies the exposure to a massive dose of the contaminant and its negative effects on health are immediate (e.g, milk contaminated with melamine). Chronic exposure implies a long term contact with the contaminants before the disease is manifested (e.g, heavy metals). An issue that has recently become a priority is related to the negative effects on normal development of foetus and infants exposed to contaminants through the food chain (e.g, *POPs*). According to the U.S. Environmental Protection Agency (EPA) Toxic Substances Control Act list (10), there are more than 75'000 known chemicals in the environment, many of which may enter the food chain. Due to the complexity and the huge amount of information, this section will be focused on some specific compounds (including some heavy metals, dioxins and pesticides) to evaluate their impact on human health.

1.3.1 Heavy metals: lead, mercury, arsenic and cadmium

Metals are natural elements that have been used in human industry and products since millennia due to their chemical and physical properties. Metals can be easily dispersed in the environment, in soil, water and air and can be very toxic even at relatively low levels of exposure; moreover they can accumulate in specific tissues of the human body.

The U.S. Agency for Toxic Substances and Disease Registry (ATSDR) produced a complete list of the hazards present in toxic waste sites according to their prevalence and the severity of their toxicity: "heavy metals" (lead, mercury, arsenic, and cadmium) are at the top of this list (8).

The lead found in food is present as salt or oxide and only a small fraction is adsorbed by humans (up to 10%). Lead toxicity can be acute or chronic. Acute intoxications are unusual but are responsible for gastro-intestinal, hematopoietic apparatus (anemia) and nervous system (convulsions) symptoms. Chronic exposure is generally manifested with anemia, which depends on the direct toxic effect of lead on red

blood cells and bone marrow. It can cause toxic effects also on the nervous system (hyperkinesia, parlysis) and renal failure. Everyone can be affected from lead toxicity, but infants and fetuses are more vulnerable to lead exposure and can suffer serious damage to the development of their nervous system and learning disabilities.

Mercury is a chemical element widely used in scientific equipment (e.g. thermometers, barometers). However, mercurous and mercuric mercury can form inorganic and organic compounds with other chemicals and can be readily absorbed through ingestion. At high levels, mercury poisoning is responsible for injuries to the lungs and the neurologic system. At lower levels, mercury poisoning is responsible for erethism (tremor of the hands, excitability, memory loss, insomnia, timidity, and sometimes delirium).

Exposure to low doses of mercury is of great concern for its effects on the nervous system development in fetuses and infants. In 1955 after the disaster in Minamata Bay, Japan, local doctors and medical officials noticed for a long time an abnormally high frequency of cerebral palsy and other child disorders in children born in the area (congenital Minamata disease). Moreover, studies in the Faroe Islands have demonstrated that, even at much lower levels, mercury exposure of pregnant women, through dietary intake of fish and whale meat, is associated with decrements in motor function, language, memory, and neural transmission in their offspring (11-12). Organic mercury, the form of mercury bioconcentrated in fish and whale meat, readily crosses the placenta and appears in breast milk.

Exposure to arsenic originates from anthropic industrial activities and the use of products such as wood preservatives, pesticides, herbicides, fungicides, and paints. In some areas of the world, arsenic is also a natural contaminant of water. Moreover, arsenic can accumulate in seafood. Once absorbed into the body, arsenic undergoes some accumulation in soft tissue organs such as the liver, spleen, kidneys, and lungs, but the major long-term storage site for arsenic is keratin-rich tissues, such as skin, hair and nails. Acute arsenic poisoning is infamous for its lethality, since arsenic destroys the integrity of blood vessels and gastrointestinal tissue and its effect on the heart and brain are huge. Chronic exposure to lower levels of arsenic results in somewhat unusual patterns of skin hyperpigmentation, peripheral nerve damage, diabetes, and blood vessel damage (13). Chronic arsenic exposure also causes a high risk of developing a number of cancers, in particular skin, liver lung, bladder, kidney and colon cancers.

Cadmium pollution (e.g, the emissions from cadmium smelters or industrial emissions and the introduction of cadmium into sewage sludge, fertilizers, and groundwater) can result in significant human exposure through the ingestion of contaminated foodstuff, especially grains, cereals, and leafy vegetables. Once ingested, cadmium is adsorbed in the gastro-intestinal tract and accumulates in liver and kidneys. Acute high-dose exposures can cause severe respiratory irritation. Lower levels of exposure are worrisome mainly for their kidney toxicity. Even without causing kidney failure, cadmium effect on kidneys can have metabolic and pathologic consequences. In particular, the loss of calcium caused by the effect of cadmium on the kidneys can be severe enough to lead to bone weakening (osteoporosis, osteomalacia) (8).

1.3.2 Pesticides

Pesticides are a class of chemical compounds used in agriculture to fight parasites and other organisms dangerous for plants, animals and humans. They are divided into different classes of molecules according to their properties as inorganic, natural organic and synthetic organics. Synthetic organic pesticides are the most used and include DDT, DDE, aldrin, dieldrin and others. DDT (diclorofenicloroetan) was synthesized in 1940 and used in agriculture against many insects. Due to its high toxicity and high persistence, the use of DDT is now banned in most countries. The major concern on the use of pesticides is related to their carcinogenic effect, their activity as endocrine disruptors and their neurotoxic effects. Epidemiological studies on workers (agriculture) in contact with these substances showed an increased risk for their health safety (14). Scientific evidence showed that many pesticides used today have neurotoxic activity. For example, commonly used organophosphorous pesticides can inhibit the acetylcholinesterase (AChE) function, the enzyme that degrades the acetylcholine neurotransmitter in the central and peripheral nervous system. Acetylcholine can then accumulate in the nervous system producing an unwanted nervous response that can be responsible for paralysis, muscle debility, convulsions and, sometimes, death (15). Moreover, the use of some fungicides (mancozeb, maneb), that are rapidly metabolized in the organism and in the environment, can generate a highly toxic product, etilentiourea (ETU), that interferes with the thyroid functions and can induce malformations in fetuses when exposed to high doses (16).

In Europe, the legislation on the use of pesticides is complex and articulated. Very recently, the European Commission officially adopted and published a new regulation setting in motion major changes in how plant protection products are placed on the market and how they are used in practice. Essentially, the new regulation will forbid some 'active substances' in pesticides. In particular, the European Parliament says, the legislation seeks to outlaw highly toxic chemicals, such as those which cause cancer (17).

1.3.3 Polychlorinated biphenils (PCB)

Polychlorinated biphenyls (PCB) cover a group of 209 different congeners, classified according to their number and position of their chlorine atom substituents. Most important, PCB are highly persistent, are globally circulated by atmospheric transport and therefore are present in all environmental media. Due to their lipid solubility and the absence of adequate metabolic pathways in the organisms, PCB tend to bioaccumulate along the trophic chains. As a consequence, PCB are major components of POPs together with polychloro-dibenzodioxins and polychloro-dibenzofurans (PCDD/Fs) (7). In general, human exposure occurs trough the diet, particularly through the ingestion of meat, fish, milk and other dairy products, while in industrial areas showing dioxin emissions the inhaled component has a greater importance. There is a lot of concern on the negative role of dioxins on human health. Dioxins toxicity has been related to different types of cancer, endocrine interference, deficit in the immune response and developmental defects in fetuses. However, studies on children indicate that the exposure of the general population to low levels of polychlorinated PCDD/Fs does not result in any clinical evidence of disease, although accidental exposure to high levels either before or after birth have led to a number of

developmental defects (18): Experimental data indicate that the endocrine and reproductive effects of dioxins should be among the most important effects in animal and humans (19,20). Nowadays, the debate is still ongoing on the real toxic effect of dioxins after low-level exposure, an issue that needs to be further investigated.

2. State of the art and interaction between basic and applied research

2.1 Food quality control

Quality is defined as any of the features that make something of a degree of excellence or superiority (21). The word "quality" is differently used in food science and technology referring to a complex concept which includes, on one side, characteristics related to nutritional, microbiological and chemical properties as evaluated from food experts (22) and, on the other side, the sensory quality of a food defined as the attributes of the food which make it agreeable to the person who eats it (the consumer). The latter involves positive factors like color, flavor and texture (23). Quality control is the sum of all those controllable factors that ultimately positively or negatively influence the quality of the finished product, *e.g,* selection of raw materials, processing, packaging, storage and distribution methods. All along the supply chain, food is exposed to numerous hazards. To prevent or mitigate most of them, the risk factors present at each phase of the supply chain must be known and an effective and comprehensive quality system must be in place. The aim of quality control is to achieve good and consistent quality standards compatible with the market for which the product is designed. Food quality control implies the control of different food processing steps to prevent the adulteration of the final product.

Some of the most important steps that need to be evaluated in food quality control are: i. agricultural materials / ingredients; ii. processing / engineering; iii. additives; iv. packaging; v. finished product inspection (24). Problems may arise in some of those phases, having a negative impact on the finished product: they are critical points (CP).

The first CP in food control concerns soil quality. In fact, food can be contaminated at a very early stage in the food chain and the contamination propagates all the way along. Soil quality can be evaluated in two distinct ways: i. as an inherent characteristic of a soil; ii. as the "health" condition of the soil (25). The former includes some parameters that reflect the potential of a soil to perform a specific function, (i.e.: plant growth and production, quality of the plants and fruits, soil natural resources). The latter includes agricultural practices that can adulterate ground functions and composition, such as manure, the use of fertilizers and pesticides, but also man-made chemicals or other contamination that usually arises from direct industrial waste discharge into the soil, percolation of contaminated water or wind contamination. The most common chemicals involved are PCBs, solvents, lead and other heavy metals. Soil quality control is performed by environmental scientists in compliance with generic guidelines that include field measurement, also using computer models, to evaluate the minimal acceptable level of a substance and eventually determine the clean up options for the contaminated soil.

In the "food processing" industry, raw materials are the main source of contamination. Stores and warehouses often make a large use of a wide range of

raw materials. Every product has one (or several) dominant raw material on which the quality of the finished product mainly depends (26). Raw materials control is another CP to ensure food quality and to be performed it requires the use of specific sampling. The formulation of the sampling type and test applied must reflect in the finished product and must be fast, simple and suited to the purpose. These tests can be chemical, physical, bacteriological or organoleptic and are usually performed in those specific laboratories that can authorise the factory to use the raw material.

Finally, food packaging control is needed in order to protect consumers from the package to foodstuff migration of harmful substances. Nowadays, packaging is an essential element in food manufacturing processes because it gives food more safety and a longer shelf life. In Europe, the Commission of European Communities (CEC) controls and establishes directives for the use of plastic packaging materials. In general, these directives are based on analytical test methods to establish the limits of plastic-package migration into food. These analytical procedures are used: i. to identify the potential migrants and their toxicity; ii. To identify the factors responsible for migration: iii. To estimate the intake of food contaminants; iv. To determine the level of contaminants in the packaging materials and in the food they have been in contact with (27).

After the manufacturing process, food quality can not be modified. Thus finished products examination can only grant acceptance to materials reaching the desired standard or rejection to materials failing to reach this standard.

Food quality control is a concept which evolves as experience and knowledge in the field grows. In the modern world, all food processing undergoes quality control, often based on discoveries derived from basic research in the field. From these observations, it can be argued that in the future there will be a possibility to generate a unique control model and, using modern data processing methods, obtain a continuous monitoring of the events during all the phases of the food production flow.

2.2 Dietary exposure to contaminants: total diet studies

From the information collected so far, it is clear that there is a general concern on food quality, contaminations, safety and effects on human health. Moreover, there is the need to put together all information coming from different sources (government, food scientists, local agencies and others) in order to define the best approach to prevent food diseases and to establish general rules for a "better food". A strong contribute in this direction comes from both basic and applied research. There are three key steps that must be considered when defining a scientific approach to food contamination as proposed in Thacker's model (Table 1) (28).

Table 1: Thacker's model

Step	Example
Hazard identification	Pesticides, Heavy metals, PCBs
Risk/source of exposure	Diet
Risk evaluation (outcomes)	Effects on human health

First, the contaminant must be identified; then, the source through which the contaminant could reach the consumers must be identified; finally. the adverse

effect (hazard) of the contaminant must be determined in order to prevent and/or protect exposed individuals. In this context, the present paragraph will analyze some examples coming from the scientific literature regarding elegant approaches applied in different countries to estimate human exposure to food contaminants trough the diet (Total Diet Study). In particular, the case of dioxins will be considered. This topic concept will be also discussed in section 5 from a different point of view.

Bilau and co-workers (29) have recently carried out an important study on three age groups of the Flemish population, adolescents (14-15 years), mothers (18-44 years) and adults (50-65 years) to determine the intake of dioxin-like compounds via animal fats or other sources, namely dairy products, added fats, fish and seafood. The study was performed by assessing the dietary intake of all the participants to the study using a semi quantitative food frequency questionnaire (30). The questionnaire has been used to estimate the daily consumption of fat-containing food items for each participant and, based on their dietary habits, the intake of fat from the different sources (meat, fish, dairy products) was determined. Contaminant concentration (dioxin and dioxin-like compounds) was measured in food items coming from the Flemish market, via the chemical-activated luciferase gene expression (CALUX) bioassay (31). To estimate the dietary intake of dioxin-like compounds in the studied population a simple approach distribution was used, combining a point estimate for contaminants concentration with the distribution of individual consumption data (32). The result of the studies shows that a large part of the three study groups exceeded the weekly "safe" intake of dioxin-like compounds and also that this intake decreased with age. Moreover, in the Flemish population fish and seafood resulted as the main source of dietary intake of dioxin-contaminants.

In another study, the same approach was used for Swedish children and adults showing that children are a vulnerable group with a daily over-intake of dioxins from food commodities in particular from fish. For this reason, the authors suggest that it should be useful to perform age-specific dietary intake assessments to protect highly exposed individuals (33). Similar studies have also been conducted in other European countries with comparing results (see section 5).

However, a key aspect emerging from this scientific work is that in most of the studies only two of the three steps foreseen by the Thacker's model have been considered: the identification of the contaminant or hazard is fulfilled (e.g, dioxins in foodstuff) and the diet is identified as the source of contamination. It lacks the proof of concept that this low-dose exposure deriving from food and assessed by the Total Diet Study is really responsible for effects on human health (see below on section 5).

In many cases, there is a general assumption that the mere presence of the contaminant will affect or be harmful for consumer safety, now or in the future. It is clear that casual exposure to high doses of contaminants generally represents a threat for human health (e.g, dioxin exposure in Seveso population). However, the low exposure impact of some contaminants, such as dioxins, is still debated. An interesting example on this specific point comes from the Food and Drug Administration (FDA) website regarding questions and answers about dioxins: Q: "What levels of dietary dioxin exposure cause adverse health effects in humans?";

A: "Known incidents of high dioxin levels in humans have resulted from accidental exposures that are not typical with dietary exposures. Despite a large body of research and data collection, there are numerous questions and uncertainties regarding scientific data on and analysis of dioxin risk. These uncertainties are unlikely to be resolved in the near future" (www.fda.gov).

3. CNR SPECIFIC EXPERTISE: QUALIFIED TEAMS, EXTERNAL COLLABORATIONS AND FUNDING

3.1 General overview on food agency: an eye on Europe and Italy

"There are certain things only a government can do. And one of those things is ensuring that the food we eat is safe and does not cause us harm." (President of United States of America, Barack Obama).

Food always had a strong influence on daily life and production/consumption of food is central to any modern society. For this reason, at the heart of any food-related topic there is the need to consider the citizen/consumer as the final "user" of the total food/feed chain and the one who needs to be protected from any risk of disease. In Europe, the main agency that controls risk assessment regarding food and feed safety is the European Food Safety Authority (EFSA): "EFSA aim is to provide appropriate, consistent, accurate and timely communications on food safety issues to all stakeholders and the public at large, based on the Authority's risk assessments and scientific expertise" (www.efsa.europe.eu). The main mandate of EFSA is related to risk assessment and risk communication. Risk assessment is a specialized field of applied science that involves the analysis of scientific data and studies in order to evaluate risks associated with certain hazards. This implies scientific data collection and analysis on a wide variety of hazards (*e.g,* pesticides, PCBs, microbiological agents and others) to gather information on dangers posed from these substances, to develop general methods to assign a date risk for the consumer. One of the key responsibilities of EFSA is to communicate food and feed safety advice to its principal clients, stakeholders and the public in a timely, clear and helpful way, in order to help bridge the gap between science and the consumer.

It is clear that due to the complexity of this issue, and, what is more, the different sources of information ranging from local to international agencies, a key step concerning food safety is the possibility to exchange information among controlling agencies. Nowadays, the Rapid Alert System for Food and Feed (RASFF) in Europe represents a powerful tool to exchange data about measures taken in response to serious risks detected in food or feed. There is a very simple principle at the basis of the RASFF system: "Whenever a member of the network has any information relating to the existence of a serious direct or indirect risk to human health deriving from food or feed, this information is immediately notified to the Commission under the RASFF. The Commission immediately transmits this information to the members of the network" (34) (Fig. 2) .

In Italy, the main actions concerning food quality and control derive from government agencies, local offices and/or private companies. Most information coming from the government is released by the *Ministero delle politiche agricole alimentari e forestali* (www.politicheagricole.it), and the National Institute of Health (www.iss.it). At local level, control and communication roles are mainly played by the Environment

Protection Regional Agencies *(ARPA)*, the Local Health Authorities *(ASL)*, the National Agrifood Informative System *(SIAN)* and others, often working in collaboration with the local police. All these agencies develop specific actions and projects to understand, prevent, control and reduce the risk related to food contamination.

Several institutes of the National Research Council (www.cnr.it) are involved in these tasks from different points of view: i. to develop new methods and strategies for analysis; ii. To apply for national and international research projects in the field; iii. To establish collaborations and consultation with official agencies devoted to control activities. In this context, one of the actions in the PIAS project has been to better categorize data in the field of "food chain contamination and effects on human health" originated from the scientific work performed by CNR research groups, an issue that will be further described in the following section.

3.2 CNR research activities: results from PIAS questionnaire

The CNR is a public organisation promoting, transferring, communicating and enhancing scientific research in different fields to improve the country's technological, economic and social activities. The organization is divided into eleven Departments, also in accordance with the research work performed at CNR. All the relevant projects developed at CNR can be viewed surfing the CNR web sites (www.cnr.it; www.cnr.it/progetti/Progetti.html).

Due to the huge amount of information and the many different scientific topics that are part of the research activities performed in the CNR, sometimes it could be very hard to gain data on the field of interest. In this view, one of the aims of the PIAS project was to clarify, and eventually harmonize, the activities of the different scientists in CNR trough communication, data exchange and eventually collaboration. In our survey, out field of interest was the "monitoring of environmental contaminants in the food chain and their impact on human health." To acquire information on the activities related to this topic at CNR, we elaborated a questionnaire which was send to the main Departments and Institutes involved

Table 2. Summary of the information obtained from the PIAS questionnaire

Methods applied	Biochemistry, GC-MS, HPLC-DAD, analytical chemistry, Gas chromatography, molecular biology, cell biology, bioinformatics, immunochemistry, epidemiology, biomarkers, mass-spectrometry
Contaminants studied	POPs, pesticides, heavy metals, organic compounds, toxins, hormones
Food	Pasta, bread, milk, cereal, fruit, vegetables, fats
Pathology	Cardiovascular disease, inflammation, reproductive fitness, cancer, neurodegenerative disease, toxinfections, lung disease, genetic disease, immune response

in our field of interest. Key points in the schedule were represented by the group composition, expertise, methodologies applied, type of contaminant/food studied, type of pathologies analyzed and main projects developed by the research groups. The data elaborated from the questionnaire are summarized in Table 2.

From Table 2, we can assume that CNR possesses specific expertise originated by the different groups working on the indicated contaminants, present in food matrices reported in Table 2 by using specific methodologies spanning from cell biology to mass spectrometry. This approach allows the performance of a risk assessment related to specific diseases. It is clear that the data reported in Table 2 probably represent only a fraction of the real competence present at CNR. This underestimation is probably due to an incomplete feedback received from the questionnaire we send. However, the data collected show the existence of the capabilities required to fulfill Thacker's model, which are already part of CNR scientists' cultural background. This is a key point, because we can speculate that, in a near future, it will be possible to develop a global, collaborative project in the field of food quality and safety related to the presence of environmental contaminants by merging the different competences coming from the different CNR groups.

4. Highlights

4.1 Heavy metals in food: traditional and innovative detection methods

Metals are constituents of the human body and some of them are fundamental for body growth, metabolic reactions and catalysis mechanisms. For this reason they are considered as *essential* constituents for life, and are distinguished in 'major' or 'minor' (trace), depending on their concentration levels (35-39).

However, the role that some metals play in the human body is not completely understood. Some metals have been recognized to be certainly dangerous for human health. Cd, Hg, Pb, As, Ni, Al, Cu may cause illness, aging and even genetic defects, and mankind is today exposed to the highest levels of these metals due to their use in industry, to the unrestricted burning of coal, natural gas and petroleum, and to the incineration of waste materials worldwide (40-48).

The term *heavy metals* is often used in current literature to indicate the toxic metals as a group, although *heavy* refers to mass (it should be actually used only for elements the atomic weight of which is higher than 200 such as mercury, thallium, lead and bismuth) and mass does not seem directly related to toxicity (49,50).

Plants contamination occurs when heavy metals are present in the soil where they are grown, and animals that are fed with these plants are also contaminated (51,52). The quantities of the different elements in soil generally vary from place to place, and the amounts absorbed by plants and retained in their tissues can also show large variations (15,53-60). Therefore, there can be considerable variations in concentrations and also in isotopic composition of metals even within the same class of food, depending on its geographical origins and other factors. For these reasons, concentration and/or isotopic ratios of metals can be used, sometimes with success, as indicators of food provenance (61-71).

Changes in concentrations and isotopic composition of metals in food may be not confined to primary, geographically-related variations. They also may be due to food manufacture, and in some cases,

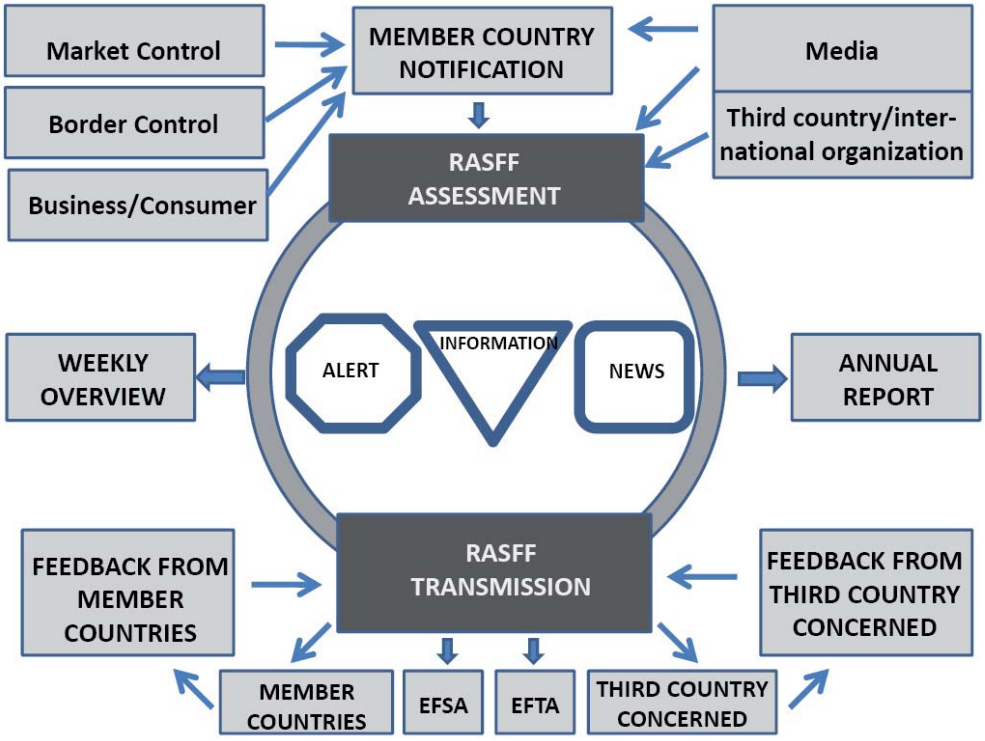

Figure 2. Schematic representation of the information flow of the RASFF

trace of metals can cause undesirable changes in food during cooking and/or storage (72-75). All these factors may have consequences on the consumers; therefore, many government authorities have specific rules for food manufacture.

In the last two decades, analytical techniques and instrumentation to determine concentrations of metals in foods have been improved.(76-80). A limited number of methods are mainly used in this field and they include: Atomic Absorption Spectrophotometry (AAS) (81-86), Spectrofluorimetry, Gas Chromatography-Mass Spectrometry (GC-MS) (87,88), Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (80,83,89-91), Inductively Coupled Plasma-Mass spectrometry (ICP-MS) (83,89,92-108), Isotope Dilution combined with Thermal Ionization Source Mass Spectrometry (ID-TIMS) (usually preceded by exchange chromatography) (109-112), with ICP-MS (113,114) and with GC-MS (115).

In some of these techniques (AAS-F/GF, ICP-AES/MS, ID-TIMS/ICP-MS), samples are reduced to a perfectly homogeneous solution before the instrumental analysis, with the exception of liquids, such as beverages (including water) which may only require dilution. In most foods, organic matter must be removed by oxidation because it would interfere with the analytical process, either by the use of oxidising acids in a wet digestion or by dry ashing in the presence of air or pure oxygen. Virtually, all organic matrices of food can undergo these two different preparation procedures and the choice depends mainly on the metal(s) to be determined. Dry ashing technique at 550°C causes Hg, Sn and As loss by evaporation. Thus, for these elements sample digestion with HNO_3

and H_2O_2 in closed PTFE vials within a microwave system is the typical preferred procedure(101,104,116-121).

AAS is a very versatile technique which does not need a particular laboratory and instrumental condition, and AAS-GF may allow determinations at ultra-trace concentration levels (<1 ppm). However, in this technique, only one element at a time can be determined, and, due to relatively low operative temperatures, determination of the refractory metals (e.g, REE, Sc and Y) is substantially precluded (83).

Due to definitely higher temperature in the torch, ICP-AES/MS is a powerful tool to determine all the metals, including the refractory ones, combining high sensibility with considerable accuracy and fastness (80,83,89-108). In particular, ICP-MS, by rapid determination of isotope ratios of elements, can be combined with isotope dilution technique in multi-collector instruments (MC-ICP-MS), greatly increasing the precision and the accuracy of the determinations (113,114,122,123).

At present, ICP-MS technique has considerably extended its capabilities, by combining with different separation procedures, as chromatography and electrophoresis, and by developing of methods of sample introduction, such as flow injection, thermal vaporisation and laser ablation (92,95-100,106,124-126).

Unlike the AAS system, however, the ICP systems require quite rigorous laboratory and instrumental conditions to improve the stability of emission (78).

LA-ICP-MS is a recent, very effective method to determine metals in food samples. Since substantial part of the sample preparation is avoided (there is no preparation except that food is dried at 110°C), when compared with classical ICP-MS, this technique is faster, and possible contamination effects due to reagents is greatly limited. Moreover, it allows accurate mapping of the analyzed sample. However, due to kinetic effects in laser ablation and/or in sample transfer to ICP-MS system, elemental fractionation is generated, so that results obtained by LA-ICP-MS technique must be considered semi-quantitative, unless the effects of fractionation can be corrected by instrumental calibration with adequate standards (101,127,128).

Isotope dilution technique may be of very high sensitivity (< 1 ppb), depending on the isotopes and on their enrichment in the tracer (spike) which is mixed to the sample that must be analyzed. Moreover, precision of the isotope dilution method in determining the concentration of a metal is related to the uncertainty which afflicts the isotopic ratio of the element which is measured for the technique. This uncertainty is amplified by a sort of magnification factor, the value of which depends on sample/spike weight ratio. Over- or underspiking of sample must be carefully avoided because they may determine magnification factor values which are significantly higher than 1 (109-112).

ID-MC-ICP-MS is greatly faster than ID-MC-TIMS but the disadvantage is the definitely lower precision both in measuring isotopic ratios and in measuring concentrations, so that ID-TIMS technique, when possible, can be considered the most effective method (61,122,129,130).

If metals must be determined at ultratrace concentration levels (<1 ppm), particular care should be taken in avoiding any possible source of contamination during sample preparation. At present, inductively coupled plasma multi-collector mass spectrometry and laser-ablation inductively coupled plasma multi-collector mass spectrometry, because of their dynamic

range and capability for multi-element analysis, are the most valuable methods for the analysis of trace elements (78,83, 90,93,101,102,104,107,108,127,128,131). Investment and operational costs for ICP-MS technique are however high, and are not justified if a limited number of elements must be determined for a limited number of samples. In this case, AAS technique or related will be preferred. Instead, for the analysis of several elements in a large numbers of samples the ICP-MS technique is economically the most advantageous. For this reason this technique is mainly used in the most important government analytical centres, where a large number of elements in a variety of food matrices are routinely determined (78,131,132).

4.2 Monitoring dioxins in food and in biological matrices by high resolution mass spectrometry

In recent years, based on a growing body of evidence, there is an increasing concern about the possible health threat posed by substances present in environment, food and consumer products termed endocrine-disruptors (EDs) and defined as "exogenous substances that cause adverse health effects in an intact organism or in its progeny, consequent to changes in endocrine function" (133,134).

The group of molecules identified as EDs is highly heterogeneous and includes synthetic chemicals used as industrial solvents/lubricants and their by-products [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides (vinclozolin), and pharmaceutical agents [diethylstilbestrol (DES)]. Moreover, some naturally occurring compounds, present in plants and termed phytoestrogens, have been found to posses estrogenic properties. The majority of phytoestrogens belongs to the large group of flavonoids. EDs have long environmental half-life resulting in a continue increase of their global concentration in the environment; furthermore, they have very low water solubility and extremely high lipid solubility, leading to their bioaccumulation in adipose tissue. Although several studies have definitively assess the toxic properties of those polluting compounds, conclusive evidences are still lacking on the effect of low doses exposition and on the synergistic effect of complex mixtures of compounds. Different studies have been performed in Germany(135), Belgium (136), Sweden (33) and Japan (137), in order to evaluate the body burden levels of PCDDs/PCDFs and DL-PCBs on general population. However, similar studies have not been performed on general population in Italy. The only data available up to now for Italian population are those regarding Seveso population who experienced the highest levels of TCDD exposure known in a residential population (138,139).

Concerning the chemical properties, PCDDs, PCDFs and PCBs constitute a group of 419 persistent environmental chemicals. Only 17 congeners among PCDDs and PCDFs and 12 congeners among DL-PCBs cause toxic responses similar to those caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic congener within these groups of compounds. PCDDs, PCDFs, and PCBs exist in environmental and biological samples as complex mixtures of various congeners with different rates of degradation due to their different solubility and volatility. Therefore, the relative concentration of congeners differ

across trophic levels and the composition of these mixtures is often very different from the one originally released into the environment. The complex nature of these chemicals complicates the health risk evaluation for humans. In order to facilitate risk assessment and regulatory control of exposure to these mixtures the concept of toxic equivalency factors (TEFs) has been developed. TEF values are used to calculate the toxic equivalent (TEQ) concentrations in various matrices (animal tissues, soil, sediment and water). TEFs and TEQs are used for risk characterization and management purposes (140,141).

In the frame of PIAS project, in a tight collaboration with the other groups participating to the project, we propose to monitor the concentration of polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin like polychlorinated biphenyls biphenyls (DL-PCBs) in blood samples from non occupationally exposed subjects as well as in selected food matrices, typical of the Mediterranean diets such as milk, mozzarella cheese, meat and fish, which, being particularly rich in the lipids, bio accumulate such molecules. For the different matrices, the concentration of some dioxins congeners (PCDDs, PCDFs, PCBs) will be determined by isotope dilution high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) (142-144).

Experimentally, after adding PCDD/F and PCB congeners isotopically labelled with the isotope ^{13}Carbon, the samples will be submitted to specific extraction and gel clean-up steps. PCDD/F/PCB concentration will be reported as pg/g fat, pg WHO-TEQ/ g fat. The measurements will be performed by means of HRGC/HRMS: the mixtures will be separated on a Gas Chromatographer, using a DB-5 capillary column, coupled on line with a high resolution double focusing mass spectrometer. The Proteomic and Biomolecular Mass Spectrometry Center is equipped with a Autospec NT instrument (Waters) specifically suitable for the analysis of dioxins and dioxin-like compounds, having a resolution higher that 10.000 FWHM. The use of high resolution capillary gas chromatography and highly selective MS conditions (select ion monitoring, resolution > 10.000, accurate m/z assignment to 0.001 Da) greatly reduces the potential for co-extracted compounds to interfere with the measurements of those analytes. Moreover, the use of PCDD/F and PCB congeners isotopically labelled allow the accurate calculation of the analytes concentration. However, it should be underlined that the determination of dioxins concentration in blood and food samples requires long and laborious analytical procedures, high cost of analysis (about 300 €/sample) as well as the use of dedicated instruments and highly expert operators.

The proposed biomonitoring will provide results of relevant importance for the estimation of the toxic human burden due to both the environmental exposure and the food chain. Information on whether and what extent chemical substances are really taken up from the environment (internal dose) are of fundamental importance for the evaluation of the related risk for human health and to elucidate the effect of low dose exposure.

4.3 Biomarkers to determine dietary exposure to xenobiotics: the case of cytochrome P450

The human cytochrome P450 (CYP) superfamily, containing 57 genes (145), contributes to the metabolism of a variety of xenobiotics including drugs,

carcinogens, constituents of food including chemicals present as pollutants (146). The resultant increases in polarity usually facilitate excretion and are considered to be a detoxification process, but in some cases foreign compounds are converted to products with much greater toxicity (147). Chemicals present in the diet may be metabolized by CYPs to non-toxic metabolites and excreted, however the formation of toxic metabolites is possible (148). It was reported that xenobiotics may be substrates, inhibitors or inducers of CYPs. Natural products present in cruciferous vegetables have been shown to selectively up-regulate CYP1A1 and CYP1A2 isozymes on chronic ingestion (148). On the other hand, several natural products selectively inhibit mono-oxygenation, especially in the intestine, and may lead to increased bioavailability and reduced metabolism of dietary components (149). CYP1A is important as it is involved in bioactivation of ubiquitous environmental contaminants such as polychlorinated dibenzo p-dioxines and in the past much concern has been focused on the induction of CYP1A as sensitive bioindicator for the exposure of fish to these contaminants in the marine environment (150). In this context, we demonstrated that CYP1A can be a useful probe for the exposure of adult sea bass and frog to polycyclic aromatic hydrocarbons (151). β-Naphtoflavone, a typical polycyclic aromatic hydrocarbons resulted in an induction in the liver of CYP1A and the induction was manifested by: i. immunoblot analysis using anti-rat CYP1A1; ii. an increase in CYP1A-mediated methoxyresorufin-O-demethylase and ethoxyresorufin-O-deethylase activities.

We also demonstrated that the CYP2A-like inhibition can be used as biomarker of exposure of herbicides, such as dichlobenil. Expression of CYP2E1 in human circulating lymphocytes has raised clinical interest because it has been proposed as a potential non-invasive bio-assay determination of CYP2E1 expression and activity in vivo (152). An elevation of CYP2E1 has been reported in lymphocytes from poorly controlled diabetic patients (153). Considering that cytochrome P450 can be induced by several xenobiotics, we can suppose that components of the diet, mainly those present as pollutants or additives, can modulate the cytochrome P450 isoforms. For this reason we can use this system as biomarker to assess dietary exposure to xenobiotics. The studies could be performed using animal models, by the administration of extract of food to evaluate modulation of CYPs. This aspect can also be investigated in humans using lymphocytes to assess if some CYP isoforms are induced and/or inhibited following ingestion of contaminants present in the diet.

4.4 Marine invertebrate as model to assay the effect of xenobiotics on reproduction

In the last decade, the international scientific community has become increasingly concerned that exposure to low levels of synthetic chemicals or xenobiotics may disturb hormone function in man and animals (so-called endocrine disruptors – Medical Research Council (UK), 1995; Danish Environmental Protection agency, 1995). During the past 50 years, large quantities of diverse xenobiotics have been released into the environment as a consequence of efforts to increase agricultural productivity and as a result of modern manufacturing processes and their by-products. These chemicals include herbicides, pesticides, fungicides, plasticizers, polystirenes, PCBs, polychlorinated dibenzodioxidins,

alkylphenoliccompounds(154),organotins, and more specifically tributyltins (TBT), used for its biocide properties as the active agent in antifouling paints (155). There is increasing evidence that these xenobiotics in the environment may disrupt the endocrine systems of aquatic life and wildlife. In addition, EDs and other food-contaminating environmental pollutants represent a high risk factors in animal reproduction (156). Such chemicals are receiving more and more attention, particularly because several compounds not specifically designed to possess endocrine activity have been shown to possess unexpected hormonal activity in a wide variety of organisms. Reproductive hormone-receptor systems appear to be especially vulnerable; in fact, some EDs can interfere with the normal mechanisms of steroid hormone action and with the embryonic development of the male and female reproductive systems of wildlife and experimental animals which in turn may affect normal reproductive functions in adulthood (154). It has been demonstrated that xenobiotics acting through steroid-dependent mechanisms, interact with estrogen receptors, androgen receptors, or with certain steroid binding proteins (ABP, SHBG). The endocrine and reproductive effects of EDs are believed to be due to their ability of: i. mimicking the effects of hormones; ii. altering the pattern of synthesis and metabolism of hormones; iii. antagonizing the effects of hormones; iv. modifying hormone-receptor levels (157). In general, the magnitude of the cellular response to hormones is dependent upon the number of receptors occupied by the hormone which in turn is related to hormone concentration. Therefore, EDs could potentially alter endocrine functions by influencing the concentration of hormones through changes in the rates of their secretion or metabolism, or by interfering with hormone action at the receptor or at other sites along the hormone signal transduction pathway. It is well known that many aspects of the reproduction in vertebrates are under the control of hormones and sex steroids and a great deal of evidence has been accumulating, showing that it may also be the case in invertebrates and fish (158-161). Several types of sex steroids have been detected in various species of invertebrates (162-164). Estradiol-17β and progesterone have been found in the tissue and hemolymph of the American lobster (165,166). In *Paeneus monodon* estradiol-17β and progesterone levels in the hemolymph, ovaries and hepatopancreas were related to the ovarian stage of development (167). Injections of progesterone and 17αOH progesterone induce ovarian maturation in *Metapeneus ensis (*168) and stimulate vitellogenin secretion in *Paeneus japonicas* (169) Estrogens stimulate vitellogenin synthesis in *Macrobrachium rosenbergii* and in *Paeneus monodon* (167,170). In the female of *Pandalus kessleri* the level of estradiol coincides with vitellogenin in the hemolymph (166). In *P. monodon* estrogen treatment during vitellogenesis may suppress molting, while stimulating vitellogenin production (167). Sex steroid hormones (androgens, progesterone, estradiol-17β) and 3 β-hydroxysteroid deydrogenase, a key enzyme in steroidogenesis, have been reported in the gonad of the male of the cephalopod *Octopus vulgaris* (171-174).

Many animal models are suitable for comparative studies with mammalian models in particular the marine invertebrate *Ciona intestinalis* (ascidians) share many common biological mechanisms with vertebrates (175). The effect of compounds deriving from marine diatoms have been

already investigated showing an influence at molecular level on the initial mechanism of fertilization. This effect seems also to influence the following embryo development (176). Similarly to ascidians, also the mollusk *Octopus vulgaris* share basic biological mechanisms with mammals. The germinal vesicle breakdown which is the first event in oocyte maturation appears to be supported by an ion current activity of specific L-type calcium channels occurring also in ascidians and mammals (177-179). At present, a study is in progress on the effects of four different heavy metals lead, cadmium, zinc and copper on the ion currents present on the plasma membrane of the oocytes and on the electrical events involved in the processes of maturation fertilization and embryo development of the ascidian *Ciona intestinalis*. Data obtained show an inhibition of either plasma membrane currents and the first events of fertilization. These results suggest a plausible negative impact of the xenobiotics on the early events of reproduction in model animals.

Although the ability of some environmental chemicals to exert toxicity on human health and reproductive fitness remains largely speculative, evidence are accumulating that multiple stressors from contaminated environment may adversely affect populations of marine animals and mammals such as humans by interfering with similar known processes of the reproductive process (58,180).

5. Future Perspectives: the effects of low-dose exposure to dietary contaminants in children and young adults: a working hypothesis

Specific goals of PIAS are to propose new projects at national and European level which may fill some of the gaps in the literature regarding the complex interaction between contaminants and human health. This working group identified the need to determine a real cause-effect relation between level of contaminants in the diet, their "real" presence in selected human populations and their effect on health. In current scientific literature, this problem has been successfully approached with excellent studies where measurements of contaminants present in the environment and bio-accumulated in the food chain have been linked to individual consumption extrapolating, from these data, the human intake in specific age groups. As already mentioned above (section 2.2), a significant example comes from the study of Bilau and co-workers (29) who report data on the dietary exposure to dioxin-like compounds in adolescents, their mothers and adults, a result of the Flemish Environment and Health Study (www.milieu-en-gezondheid.be). They demonstrated that in the selected aged groups, the median (95^{th} percentile) estimated daily intakes of dioxin-like contaminants were 2.24 (4.61), 2.09 (4.26), and 1.74 (3.53) pg CALUXTEQ $kg^{-1}bwd^{-1}$ for, respectively, adolescents, mothers and adults. These values exceed the tolerable weekly intake (TWI) of 14 pg WHO-TEQ $kg^{-1}bww^{-1}$, as derived by the Scientific Committee on Food (181). The relative validity and reproducibility of this experimental approach was assessed by the same authors in a different study (182). Here, they concluded that the food frequency questionnaire designed to estimate the intake of dioxin-like contaminants represents a valuable tool for ranking individuals in the study population on the basis of estimated intake of dioxin-like contaminants. However, absolute intakes should be estimated without correction factors and interpreted with caution. In a similar study carried

out by a Swedish group within the EU funded CASCADE Network of Excellence (Contract N. FOOD-CT-2003-506319) the dietary intake of dioxin-like pollutants was investigated in children and young adults (33). The results showed that among the selected Swedish population, boys and girls up to the age of ten years had a median TEQ intake that exceeded the tolerable daily intake (TDI) of 2 pg TEQ/kg body weight. Dairy and fish products were the main sources of exposure. In fact, the individuals most highly exposed were characterized by a high consumption of fish. Also in this case, exposure was estimated matching the concentration data of dioxin-like compound in food commodities (meat, fish, dairy products, egg, edible fats and other foodstuff) with food intake data. Similar studies on dioxins exposure via food were performed in several countries, generally showing that estimated dietary intake is above the recommended TDI level ranging from roughly 2–6 pg TEQ/kg bw/day (183-192). On the opposite, an Italian study established that the mean value of dioxins measured in food of animal origin by isotope dilution method was 0.144 ± 0.266 pg-TEQ/g (range: 0.003–1.655 pg-TEQ/g). The average daily food intake was obtained from national data collected by the National Institute of Nutrition, and from a cohort study on diet and cancer including 40,000 Italian subjects. The conclusion was that the major contribution to dioxins intake with food comes from cow milk and fish consumption and were below the limits set by the European legislation (193). Apparently, the adherence to the limits established by EC (194) was confirmed in parallel studies carried out in different Countries such as Germany (195), Finland (196), Japan (197) and Spain (198).

As discussed by others (193), many of the studies cited suffer from the same limitations: i. the amount of dioxins, or other contaminants, intake with diet was estimated from national surveys or epidemiological studies, without measuring dioxins content of a certain food and the individual intake of that foodstuff; ii. data were obtained through a monitoring program, not as part of a research project; this means that the aim of the monitoring was not to study human exposure through food, but to assess food content of dioxins and other residues; iii. dioxin content was lacking for certain food consumed within the population, making the analyses incomplete.

Based on this preamble, we proposed within the PIAS project a large, multicenter and multidisciplinary study with an extended follow-up which will take into account the missing information existing in the Literature. The target population will be represented by children and young adults. In fact, they constitute a vulnerable group and previous studies suggest that it is essential to perform age-specific dietary intake assessments to more carefully consider, in the risk management processes, sensitive and/or highly exposed individuals in the population.

The general objective of this proposal is: *to address the healthy status of a young population determining the concentration of xenobiotics which come to humans through the food chain.*

The proposal consists in two phases:

Phase I

The working hypothesis is illustrated in Fig. 3. The experimental aim is to study populations of children, adolescents and adults living in various Italian regions, including Campania (South Italy) establishing biological banks (mainly blood and urine samples). These individuals will be selected from large Italian cohorts that are at least in part already available

from ongoing European projects involving members of PIAS working groups. The Italian study sample should be composed by about 2000 individuals with males and females equally represented. Additional cohorts with similar features from other Countries potentially interested to participate will be enrolled in the study to obtain comparable information at European level. At baseline the following variables will be measured: in complete anthropometry including body composition measures; biochemical measures, including selected hormones; physical activity tests; medical history, behavioral and socio-economic questionnaires; food-frequency questionnaire and repeated 24h dietary record. All these variables will be measured again in the same population in the follow-up survey.

Biological samples, preferentially blood and urines, freshly collected or, where possible, already available if conveniently stored for the expected analyses, will be employed to measure the presence of those contaminants whose presence was independently and previously verified in food commodities taken directly from or through the food chain. A careful evaluation of different types of contaminants/xenobiotics to be analyzed in the present project is actually under scrutiny from members of PIAS working groups. The selection will certainly include compounds belonging to the following categories: pesticides, dioxins and dioxin-like molecules and heavy metals. For the choice, two main criteria will be followed: i. presence of these compounds in the diet of the selected individuals; ii. availability of official methods to detect them in biological samples and foods.

At the end of this phase of the study, we will relate the level of contaminants present in foods and biological samples with epidemiological data from the populations under study (dietary habits, health status) to determine the potential association between concentrations of selected contaminants and health effects.

General methods. In order to assess the exposure in children and young adults, the individuals will be stratified by gender and age. Individuals with incomplete information on body weight or food consumption will be excluded. The 100 most commonly consumed food items will be collected and analyzed by standard methodology to assess the presence and concentration of selected contaminants. Food items will be obtained from producers or purchased from different stores in the cities where the cohorts will be recruited. Accordingly to reports periodically published by EC (199-201), the food groups chosen for the study will be: i. fish, dairy products, egg, edible fats and other fat-containing products for the presence of dioxins, dioxin-like molecules and selected metals; ii. cereals, fruits, vegetables, beverages, vegetable soups and sugar for the presence of pesticides, biocides and heavy metals. Exposure to different xenobiotics based on consumption of various food items by each individual will be expressed accordingly to international units establish for each specific contaminants. Data will be analyzed by standard statistical methods. In particular, dioxins concentration in different biological samples and in food groups will be associated with selected health outcomes after adjustment for age and gender. The dioxin levels in different food groups (fish, meat, dairy products, egg, edible fats, other fat-containing products, fruits, vegetables, cereals, etc.) will be compared and related to the individual intake of each food item to assess the principal sources of exposure both at the individual and at the population level. The

proposed sample size of 2000 individuals is large enough to allow statistical power for different series of analysis. For instance, it will allow to detect a 5% difference in the amount of ingested contaminants between groups, at alpha =0.05 and beta=80%.

Phase II

Information obtained from doses of contaminants in biological samples and above the threshold established by international health agencies will represent the starting point for phase II of the proposal devoted to determine whether and how, from a molecular point of view, exposure to these xenobiotics may interfere with the normal physiological state of the cell/organism resulting in pathological conditions in adults. As schematically represented in Fig. 3, phase II will take advantage of different cellular and animal experimental models suitable to address cause-effect relation in specific chronic diseases potentially associated to exposure and/or accumulation of dietary contaminants. Decision about the diseases on which to focus our attention will strictly depend upon results obtained after phase I. In choosing the research groups to assign these specific tasks, we will primarily consider expertise and competence within CNR, as resulted from the census questionnaires settled down during the course of PIAS program (see section 3.2 above). Phase II will also takes advantage of the work and competence deriving from other working groups within PIAS (e.g, endocrine disruptors). As an example of activity performed during phase II of the project, great importance will be devoted to assess the potential effect of xenobiotic exposure to reproductive fitness and development (see section 4.4) and to the role of cytochrome P450 in metabolizing xenobiotics (see section 4.3).

Key issues to be addressed in order to correctly evaluate and interpret data deriving from the experimental models employed in phase II concern: i. genetic background; ii. adaptive response/ hormesis; iii. bioavailability and metabolism of different xenobiotics.

Risk estimates routinely reflect numerous sources of both uncertainty (which describes the range of plausible risk estimates arising because of limitations in knowledge) and variability (which describes the range of risks arising because of true differences). Among them, and besides age and gender, genetic differences among members of the population may play a relevant role. Since the majority of the study on dioxins have been conducted using genetically homogeneous inbred mice to characterize the risk, their conclusions should be taken very cautiously when applied to the genetically variable human population. Although well-designed occupational and environmental epidemiological studies can yield useful information on human population variability, relatively little quantitative information is available about the potential impact on genetic polymorphisms in the human population that might give rise to differences in susceptibility to the toxic effects of dioxins, and DLCs. As an example of candidate gene, the Aryl hydrocarbon receptor (AhR or AHR) is a cytosolic transcription factor able to bind to chemicals such as TCDD, leading to changes in gene transcription. A state of the art revision of the literature will be done in the course of this project to identify candidate genes or biological pathways to be explored with genetic studies. The panels of SNPs in selected genes will be genotyped in the laboratories of ISA-CNR, using up-to-date genotyping technologies. At the current status, allelic discrimination will be performed by TaqMan® genotyping assay. The use of

other techniques like SNPs array will be considered taking into account the number of samples/SNPs to be evaluated and the cost of the assay at the time of genotyping. The genotyped SNPs will be uploaded in a central database and linked to the phenotype data. To minimize the population stratification bias, a potential source of false positive associations in genetic population studies (202), genetic analyses will be restricted to individuals of Caucasian origin. Additionally, it is likely that all the cohorts will belong to single countries, with the majority of participants coming from specific delimited geographical areas, thus further reducing the risk of population admixture.

Hormesis is a biphasic dose-response phenomenon characterized by a low-dose stimulation and a high-dose inhibition resulting in a U- or inverted U-shaped dose response (203). The phenomenon of biphasic dose-response relationship has received considerable attention over the past few years (204). A good example on the application of hormesis phenomenon to human health derives from exposure to heavy metals, such as lead, cadmium, mercury and arsenic. Cadmium is a potent carcinogen in a number of tissues, and is classified by IARC as a human carcinogen (205). Reactive oxygen species (ROS) are often implicated in cadmium toxicology, either in a variety of cell culture systems (206-209), or in intact animals through all routes of exposure (210-213). However, in contrast to acute toxicity, the roles of ROS in chronic cadmium toxicity and carcinogenesis have been controversial depending on experimental conditions. On the other hand, administration of cadmium to animals at low levels for one year increases hepatic and renal glutathione levels, without elevations in tissue lipid peroxidation levels (214). A biphasic ROS response to cadmium exposure through the drinking water has also been proposed. ROS and ROS-related gene expression occur right after cadmium exposure, but return to normal levels after 8 weeks of exposure (215). A further example comes from chromium (VI), a well-known mutagen and carcinogen that produces ROS during formation of reactive chromium intermediates (216-218) and induces oxidative stress (219). ROS are known to be generated in various cell types, such as K562 leukemic cells, J774A.1 murine macrophages (220) and human epithelial like L-41 cells (221) when acutely exposed to chromium (VI). A potential adaptive response were obtained when immortalized rat osteoblasts (FFC cell line) and U937 were exposed to 0.05-0.5 micromolar chromium (VI) for 4 weeks (222). In addition proteomic analysis of both FFC and U937 cells exposed to 0.5 micromolar chromium (VI) resulted in a differential time dependent regulation of glycolytic, stress and cytoskeletal proteins that play an essential role in normal cellular functioning such as energy metabolism, cell signaling and proliferation (223).

Hormetic responses to xenobiotic exposure likely occurring as a result of overcompensation by the homeostatic control systems operating in biological organisms have been discussed in excellent reviews which commented on the economic implications of hormesis (203,224-231).

Bioavailability refers to the extent to which humans and ecological receptors are exposed to contaminants from soil, or sediment directly or through the food chain (an extensive and excellent review on this topic has been published by the Committee on Bioavailability of Contaminants in Soils and Sediments of the National Research Council)(232). Our interest in determining the bioavailability

of potentially contaminants adsorbed with the diet is related to their risk assessment for human health. However, the concept of bioavailability has recently been exploited by hazardous waste industry as an important consideration in deciding how much waste can be left in place without creating additional risk if these contaminants are not bioavailable (232). In terms of effects on human physiology, once absorbed, contaminants may be metabolized, excreted, or they may cause toxic effects. Several levels of uncertainty are associated with bioavailability, including: i. limited knowledge about how biota modify bioavailability of chemicals which come into contact with digestive systems, and whether information obtained for one species is representative of others; ii. the effect of food processing (cocking, pressing, etc.) which makes extremely difficult to precisely calculate the daily intake of xenobiotics, despite the nominal values determined in the single food component; iii. the synergic or antagonistic effect on absorption and metabolism of xenobiotics by other food components, such as polyphenols. In this contest, literature reports examples in both directions: the protective effect of oral resveratrol on the sub-acute toxic effects of TCDD in C57BL/6J mice (233), or the confounding activity of contaminating metals which may interfere with the regulated absorption, distribution, and excretion kinetics of essential metals (234), although this study conclude that food contaminations with metals are too low to have an impact on the bioavailability of essential metals. In general, a higher priority could be given to studies exploring combinations of nutrients, xenobiotics and food contaminants, at realistic intestinal concentrations, with hazardous or beneficial impacts on human health using high throughput *in vitro* tools (235).

6. Conclusion

"We are what we eat," says Ayurveda, the ancient Indian science of life. This sentence immediately clarifies the perception of how food can influence our lives and the relevance of key issues as food safety on human health. In this Chapter, we reviewed critical aspects concerning food contamination and its impact on health. Our analyses showed that several gaps and uncertainties remain, despite the great concern for "food integrity" and the variety of scientific contributions from different fields, such as environmental science, food chemistry, human epidemiology. These limits cannot be bypassed only with the efforts of the scientific community, but the contribution of Governments and international environmental and healthy agencies is mandatory to face and rapidly solve these deficiencies. This is a critical issue: in evaluating food safety, no gaps are allowed since bad or incomplete information may result in an enormous hazard for consumer's safety and for the negative consequences that the presence of a contamination in the food chain may generate to the social and economical life of a region.

To correctly approach the issue of the negative influence (if any) of chemical contaminants reaching people throughout diet, we underlined the need of an integrated approach which considers key factors, such as the genetic background of exposed subjects, adaptive responses, age dependent accumulation and bioavailability of specific compounds. Very recently, the publication of the new European regulation concerning the use of pesticides in agriculture (17), raised the problem of the relation existing between exposure to

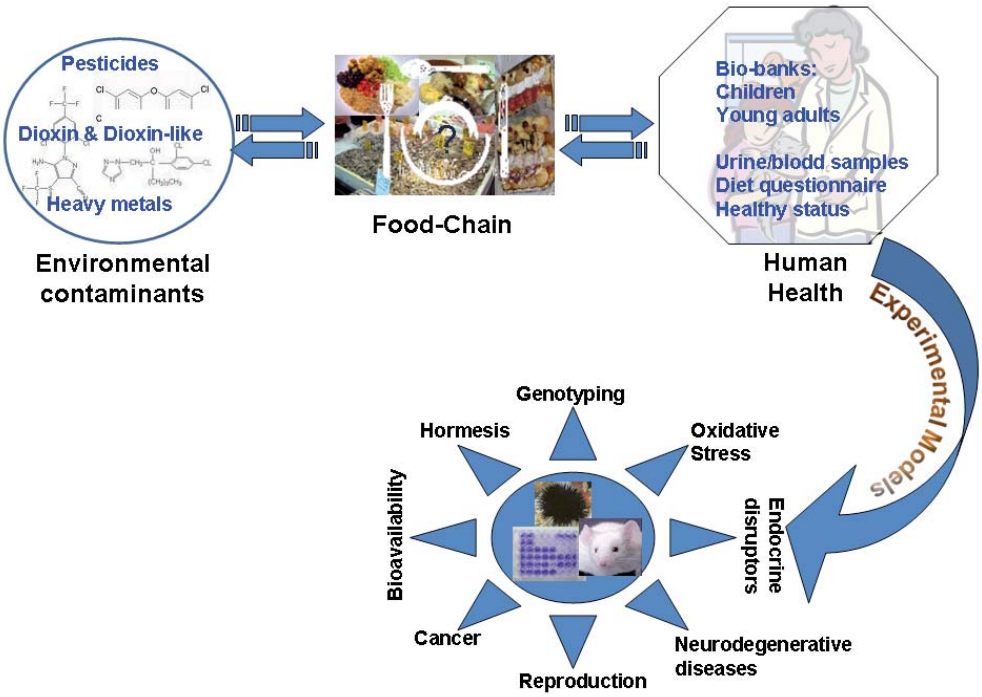

Figure 3: Schematic model of research program proposed within PIAS

low dose contaminants and their potential harmful effects in developing chronic pathologies. Several validated experimental models exist to study the consequences on human health of acute exposure to elevated concentration of chemical contaminants. On the opposite, a general consensus is still far to be reached on how to assess the effect of prolonged, low-doses exposure to environmental contaminants. Perhaps, a lessons may derive from studies in the filed of radiation exposure.

In this context, PIAS proposed a large, multidisciplinary research project aimed to fill, at least in part, the lack of information existing in the field. The realization of this study cannot be obtained solely by expertise already present within the CNR, but requires the strong contribution of national and European groups with proved experience in the numerous and different fields considered by the proposal (Fig. 3). The realization of an integrated approach to assess the impact of food contamination on human health represents, in our view, the only correct way to increase scientific knowledge and build trust and confidence in the consumers' beliefs.

Keywords: heavy metals, pesticides, dioxins, xenobiotics, food chain, human health, bioavailability, hormesis.

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