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EUROTOX

Toxicology course

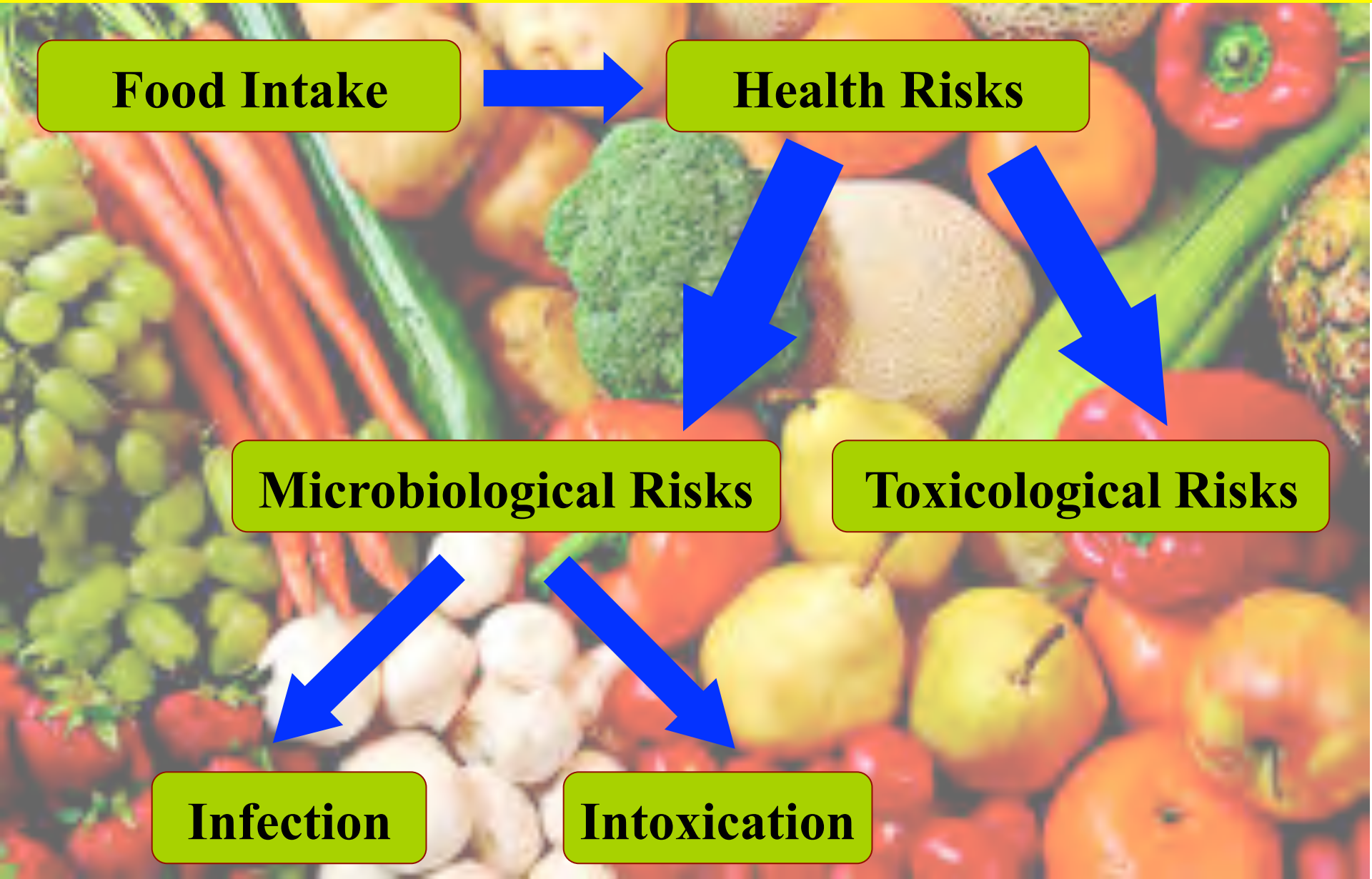
Principles of Toxicology

April 4-8, 2016

Zagreb, Croatia

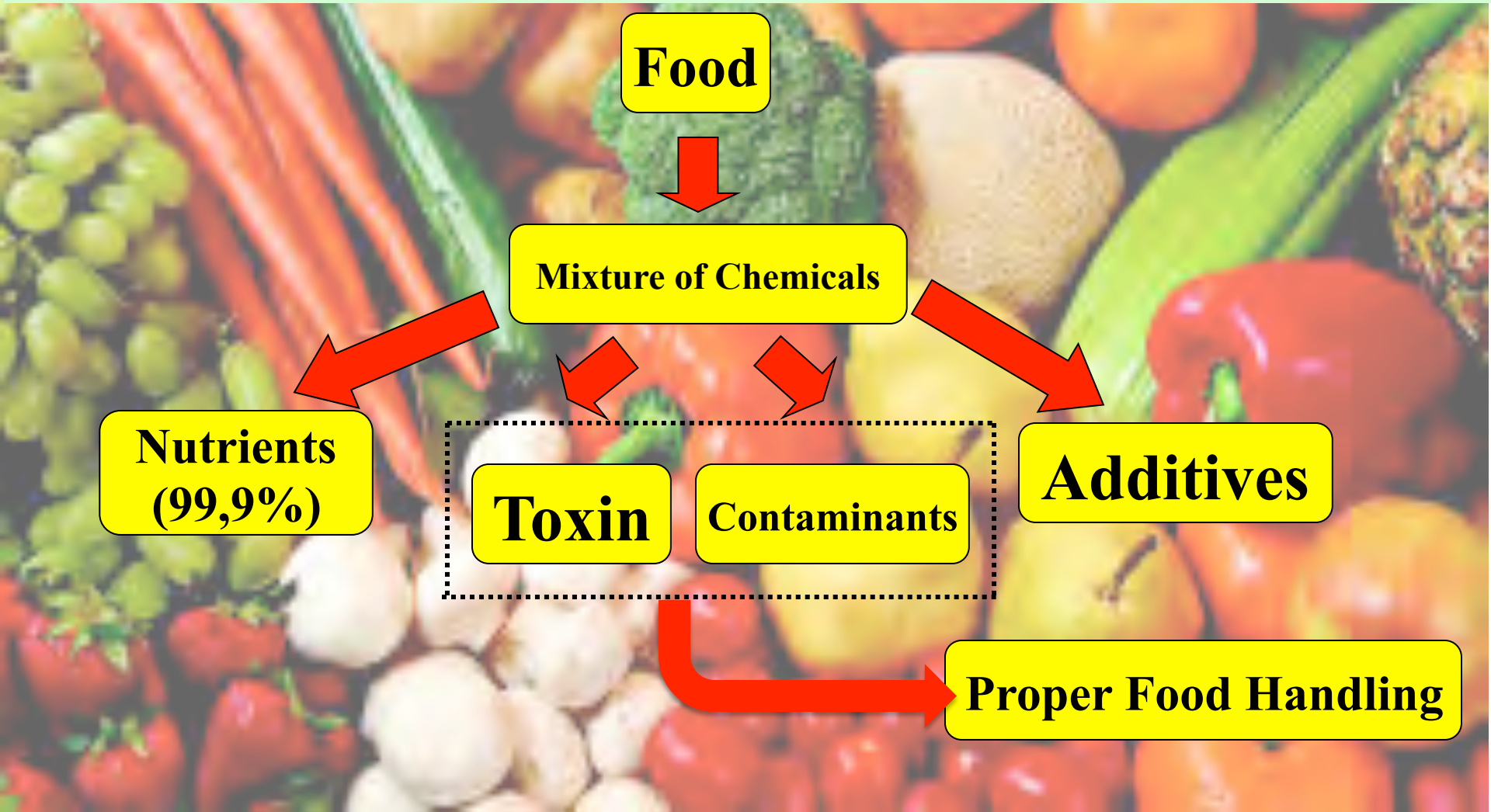
Introduction to Food Toxicology

Food Toxicology



Food Toxicology

- Food is fundamental importance to life
- Human consumes 30 tons of Food during his lifetime



Food Toxicology

Natural Toxins

A. Endogenous Toxins of Plant Origin

B. Natural contaminants

- **edible plant + toxin**
- **toxic substances by animal**
- **microbial toxins (bacterial toxin, mycotoxin)**

Definition

Food contaminants are substances included unintentionally in Foods

Some are harmless and others are hazardous because of the toxicological risks from their intake to the consumers

Definition

- **Contamination can occur at every step on the way from raw material to consumer *(from farm to fork*  *)***
- **Raw material → environmental pollutants (pesticides, toxins, chemicals, heavy metals)**
- **During processing → filtering and cleaning agent, industrial plants**
- **During packaging & storage steps (plastics, coating, toxins, tins, etc.)**

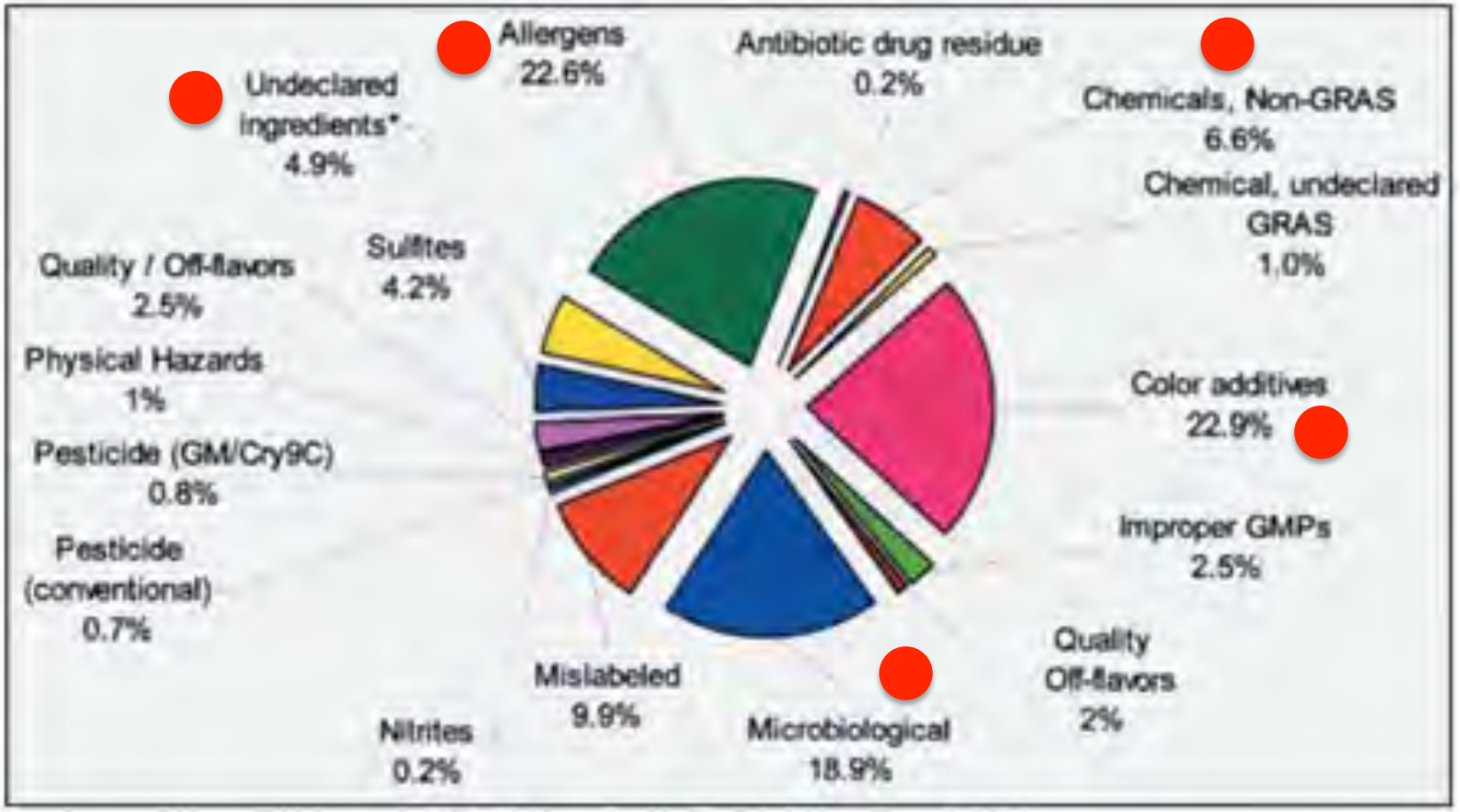
Contaminants

1. **Heavy metals**
2. Nitrate
3. **Dioxins**
4. Mycotoxins
5. **Pesticide residues**
6. Packaging materials
7. **Biotoxins**

Kinds of Food Contaminants

- **Biological**
  - bacteria
  - mold
  - fungus
  - parasites
  - viruses and other toxins
- **Chemical**
  - accidental contamination with chemicals throughout the product chain
- **Physical**
  - accidental contamination with objects due to employee carelessness

Kinds of Contaminants

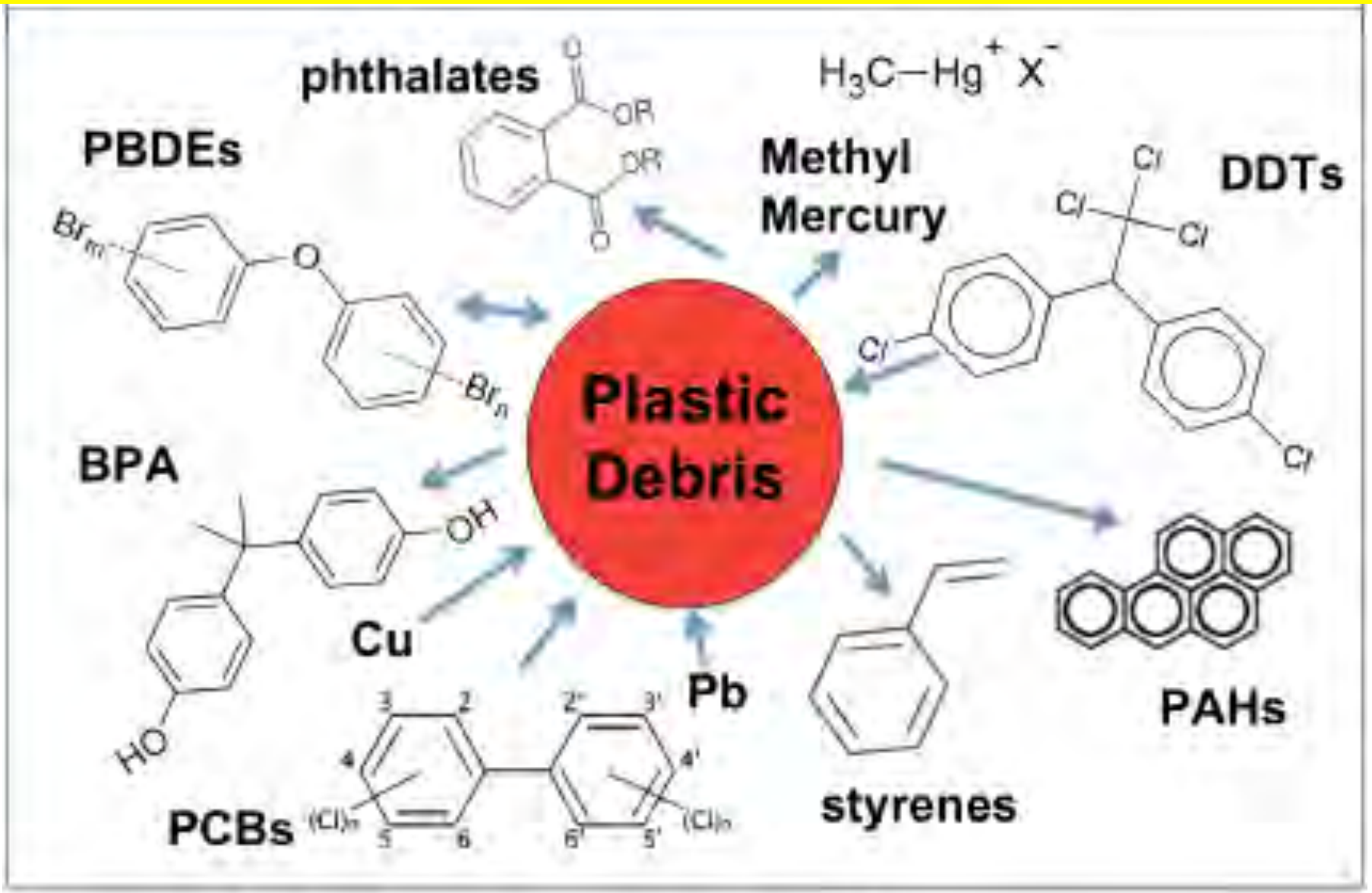


FDA food product recalls, 2001

** "Undeclared ingredients" does not include allergenic ingredients*

*** Filth contamination field as "Improper GMPs"*

Cocktail of Chemical Contaminants



Units Used to Measure Chemicals in the Food Analysis

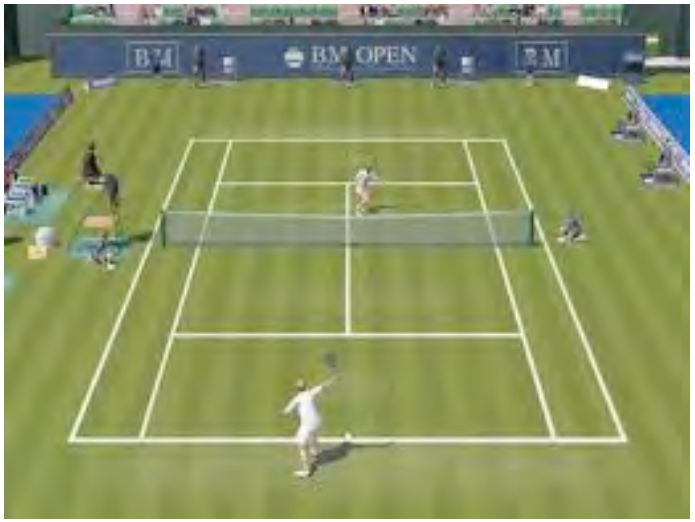
- PPM – Parts per million
- PPB – Parts per billion
- PPT – Parts per trillion

One part per million

- 2,54 cm in 25,75 km
- 1 minute frame in two years
- 1 cent in $10,000
- 31 g of salt in 31 tons of potato chips
- 1 bad apple in 2,000 barrels of apples

One part per billion

- 2,54 cm in 25,750 km



- 1 second frame in 32 years
- 1 cent in $10,000,000

- 1 pinch of salt in 10 tons of potato chips
- 1 lob in 1,200,000 tennis matches

- 1 bad apple in 2,000,000 barrels of apples

One part per trillion

- 1 postage stamp in the area of the city of Dallas (1,000 Km^2)
- 2.54 cm in 26 million Km (more than 600 times around the earth)
- 1 second frame in 320 centuries
- 1 flea on 360 million elephants

- 1 grain of sugar in an Olympic sized pool
- 1 bad apple in 2 billion barrels

POISON!!!

- **Legally defined term**

  not just anything you don't like
- **Any pesticide with an LD_{50} of 50 mg/kg or less**
- **Labels must reflect this classification**
- **Label must have the signal word "DANGER" plus the word "POISON"**
- **Label also must display the skull and crossbones icon**

GRAS Definition !!!

GRAS is an acronym for the phrase Generally Recognized As Safe. Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (the Act), any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive

Heavy Metals

1. Mercury (Hg)

The widespread use of mercury and its derivatives in industry and agriculture has resulted to **increased levels of mercury in foods**

ex: **Fish products in particular can be contaminated with mercury, as methylmercury accumulates extensively in fish**

- The primary target for mercury is the **central nervous system**

- **Case:**

– **In Iraq, the poisoning appeared to result from the ingestion of wheat treated with a fungicidal based on mercury**

– **Daily intake of mercury per individual in the US and in Western Europe is estimated at 1 to 20 µg**

The Tolerable Weekly Intake (TWI) is 300 µg

Heavy Metals

- **Source:**
  - **Natural sources: volcanoes**
  - **Gold production**
  - **Cement production**
  - **Battery production**
  - **Disposal of certain product**

Product containing mercury:

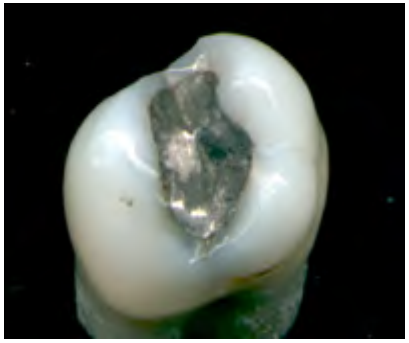
battery, medical products, thermometer

Case:

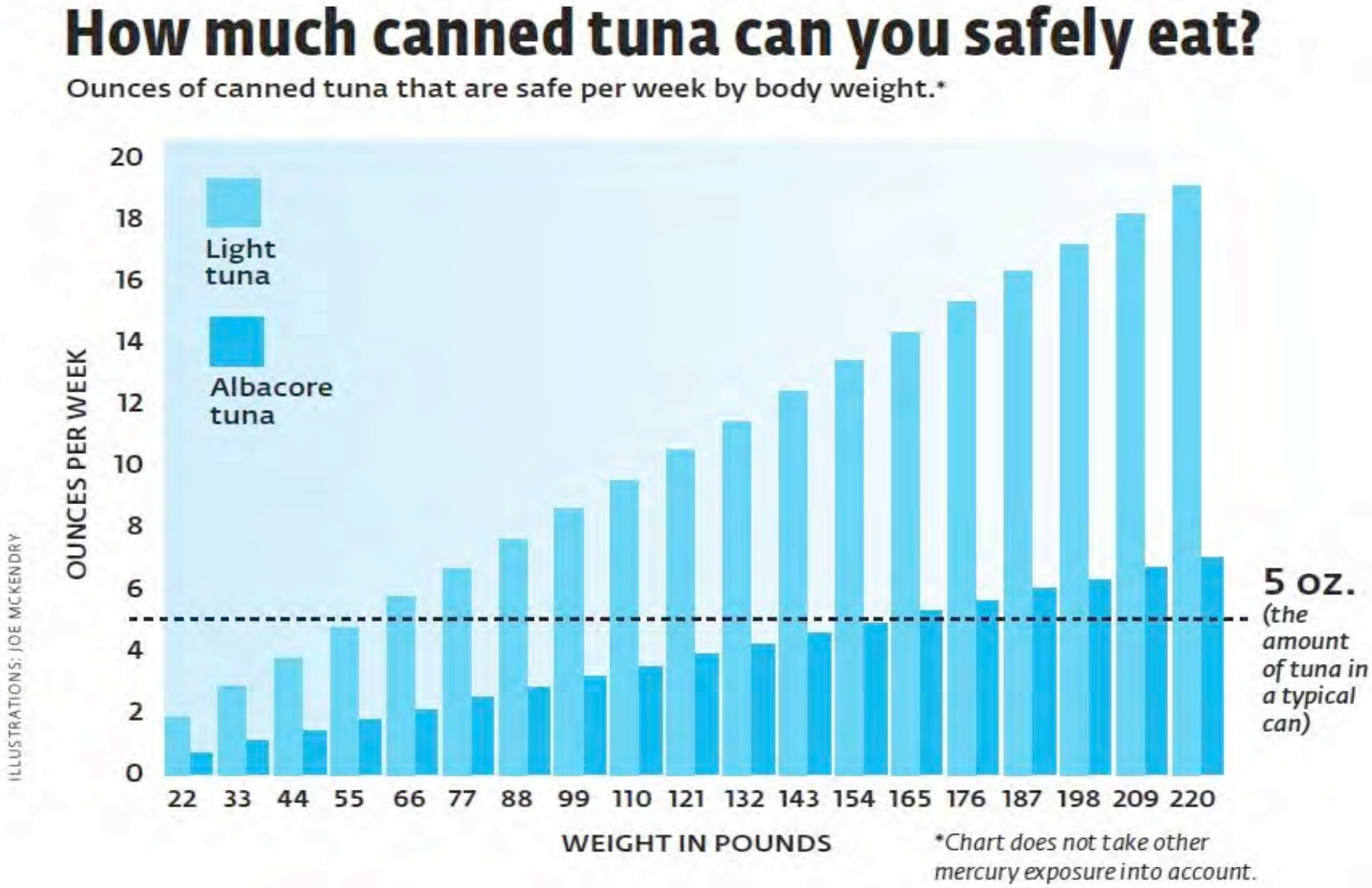
Environmental disaster caused by industrial pollution of the bay with mercury → Minamata disease is a neurological syndrome caused by severe mercury poisoning

Heavy Metals

- Symptoms include numbness in the hands and feet, general muscle weakness, narrowing of the field of vision and damage to hearing and speech, paralysis, coma and death
- Present use: medicine, amalgam filling, mascara, vaccine preservative, fluorescent lamp, NaOH production, gold mining



Mercury


How much canned tuna can you safely eat?
Ounces of canned tuna that are safe per week by body weight.*
OUNCES PER WEEK
20
18
16
14
12
10
8
6
4
2
0
Light tuna
Albacore tuna
5 oz.
(the amount of tuna in a typical can)
22 33 44 55 66 77 88 99 110 121 132 143 154 165 176 187 198 209 220
WEIGHT IN POUNDS
*Chart does not take other mercury exposure into account.
ILLUSTRATIONS: JOE MCKENDRY

Regulation European Union

- Certain electronic products: no Hg
- Other products: < 1,000 ppm
- Battery: 5 ppm
- Thermometer, Barometer: no Hg

- Fishery products and muscle meat of fish, excluding species listed in 3.3.2 (tuna etc.) of 1881/2006. The maximum level applies to crustaceans excluding the brown meat of crab and excluding head and chest lobster and similar large crustaceans (Nephropidae and Palinuridae)

0.50 ppm (in tuna the limit is 1.0 ppm)

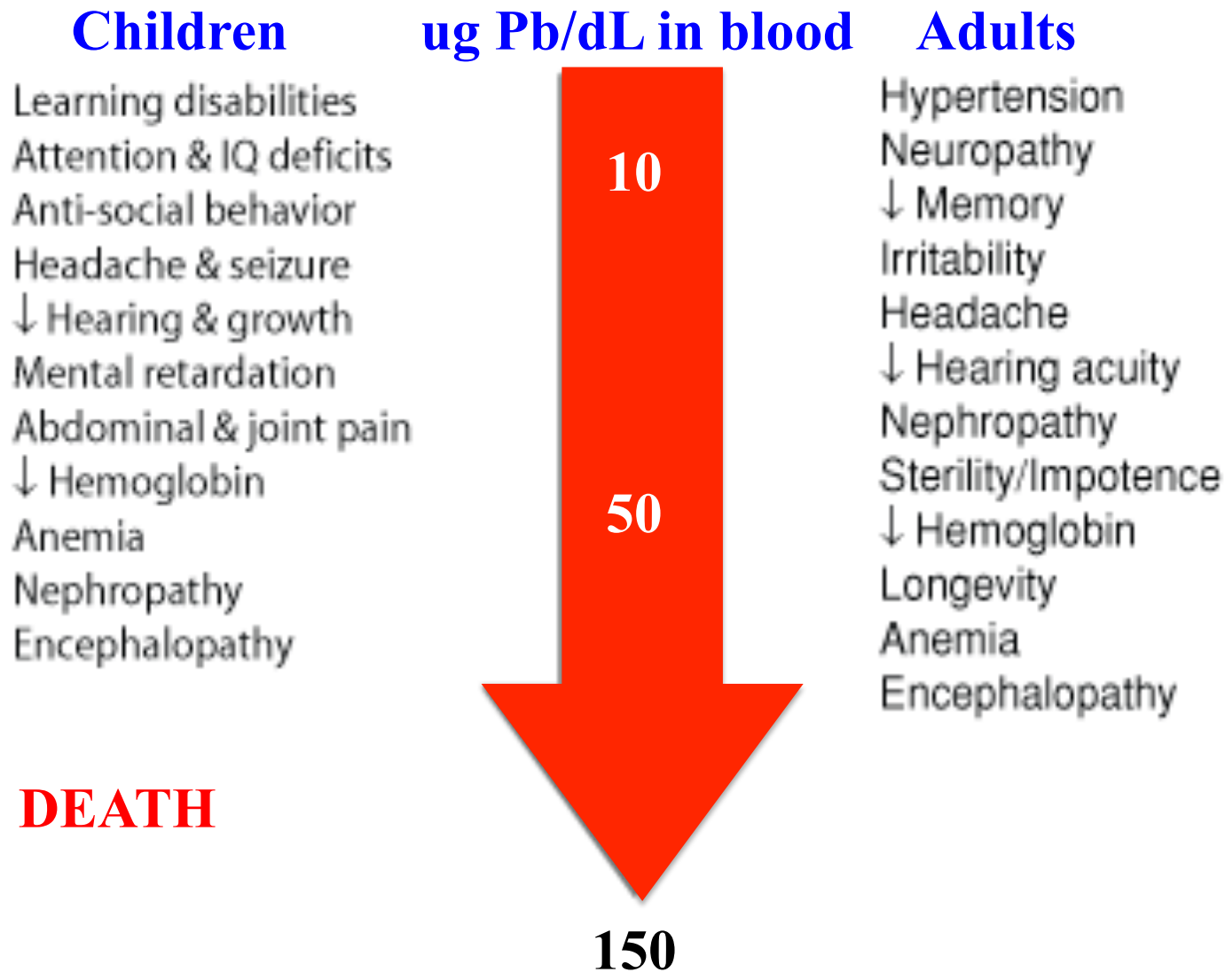
Levels of Mercury in Food

FOOD	Hg in µg/kg (ppb)		
	United States	United Kingdom	Japan
Cereal (grains)	2–25	5	12–48
Bread and flour		20	
Meats[a]	1–150	10–40	310–360
Fish[b]	0–60	70–80	35–540
Dairy products			
Milk	8	10	3–7
Cheese	80	170	–
Butter	140	10	–
Fruits	4–30	10–40	18
Vegetables (fresh)	0–20	10–25	30–60
Canned	2–7	20[c]	0
Eggs			
White	10	ND[d]	80–125
Yolk	62		330–670
Beer	4		

Lead (Pb)

- Lead (metallic gray) is a poisonous substance to animals. It damages the nervous system and causes brain disorders
- Because lead is resistant to corrosion it is extensively used in building construction – for example in the external coverings of roofing joints

Effects of Lead on human

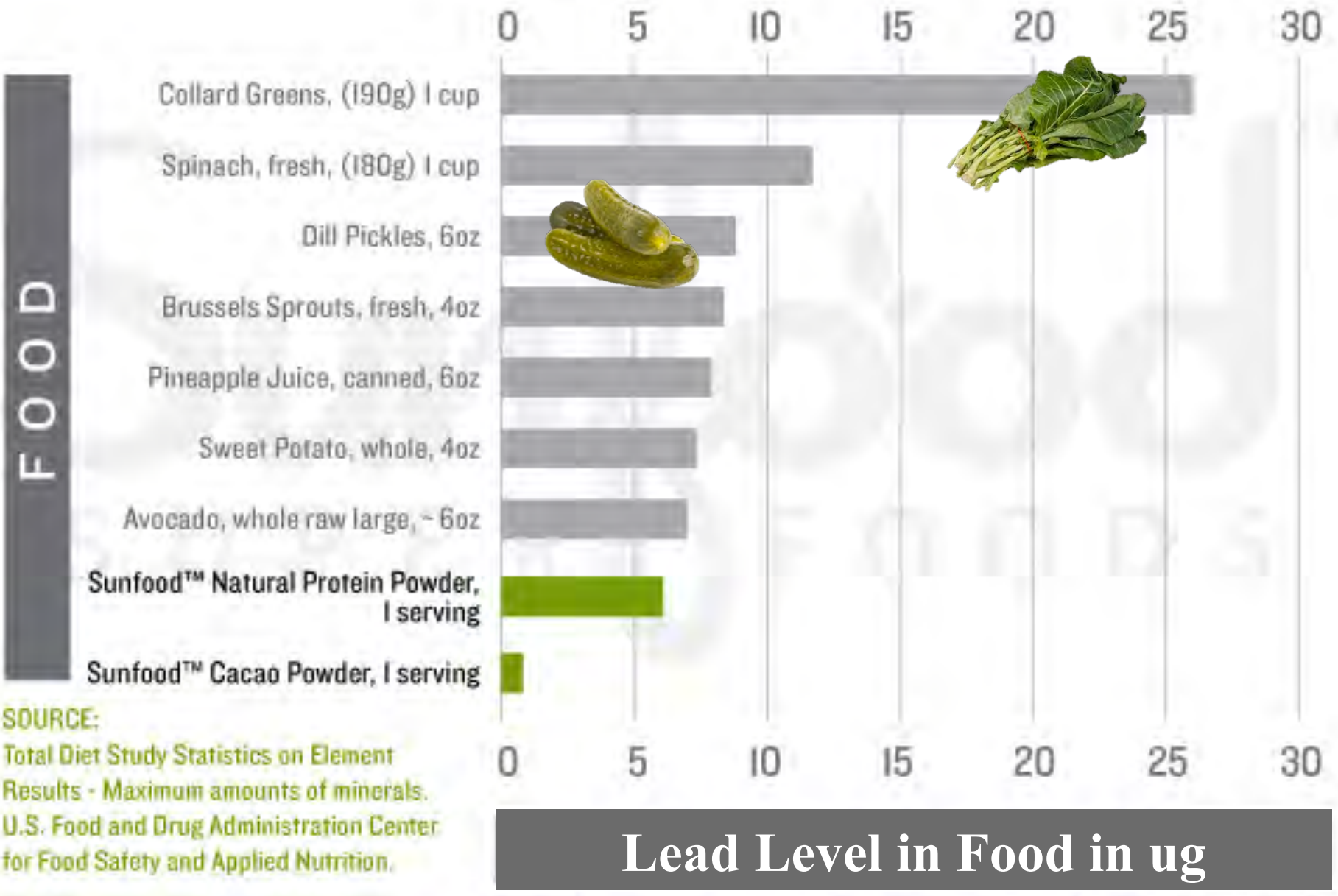


[8] Adapted from Gurer and Ercal (49).

Lead (Pb) in Food

Food	Pb, µg/100 g Range	Mean	Food	Pb, µg/100 g Range	Mean
Cereal grains	0–62	22	Cider, apple		90 µg/l
Cereal grain products	0–749	10.5	Vinegar, cider		100 µg/l
Seafood, raw	17–250	62	Cola (2 samples)	18–65 µg/l	
Canned	6–30	16	Ginger ale		10 µg/l
Meats	7–37	19	Beer, canned		40 µg/l
Gelatin		57	Wine, red		50 µg/l
Eggs, whole	0–15	7	Sugar, white	0–7	
Vegetables, leafy	0–126	37	granulated		
Legumes, raw, dried	0–16	7	Molasses		53
or frozen			Backing powder		150
Canned	3–11	7	Yeast, dry		117
Apple, raw		38	Black pepper		40
Pear, raw		3	Cinnamon		11
Milk, whole, fresh		0	Nutmeg		41
Skim, dried and packaged		2	Allspice		64
Skim, bulk package		2	Chili powder		18
evaporated	4–5	4.5	Bay leaves		55
Tea, leaves		1.37			
Cocoa, dry		0.10			

Mean Lead Levels in Food



SOURCE:
Total Diet Study Statistics on Element Results - Maximum amounts of minerals.
U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition.

Regulation

- **FDA: 8.1 μg/day, TWI→ 25 μg/kg body,**
  drinking water < 5 ppb
- **Application: solder for electronics, customization of tennis racket, construction industry (roofing materials), PVC, lead paint, lead containing gasoline**
- **Toxicity→ blood pressure, anemia, brain damage, kidney damage, misscarriage, reduce of fertility of males**
- **In Europe the limit is 0.02 ppm in milk and in baby food, while is 0.1 pp in meat and 0.2 in cereals**
- **Finally, the limit is 1,5 ppm in bivalve mussels reg. EU 1881/2006**

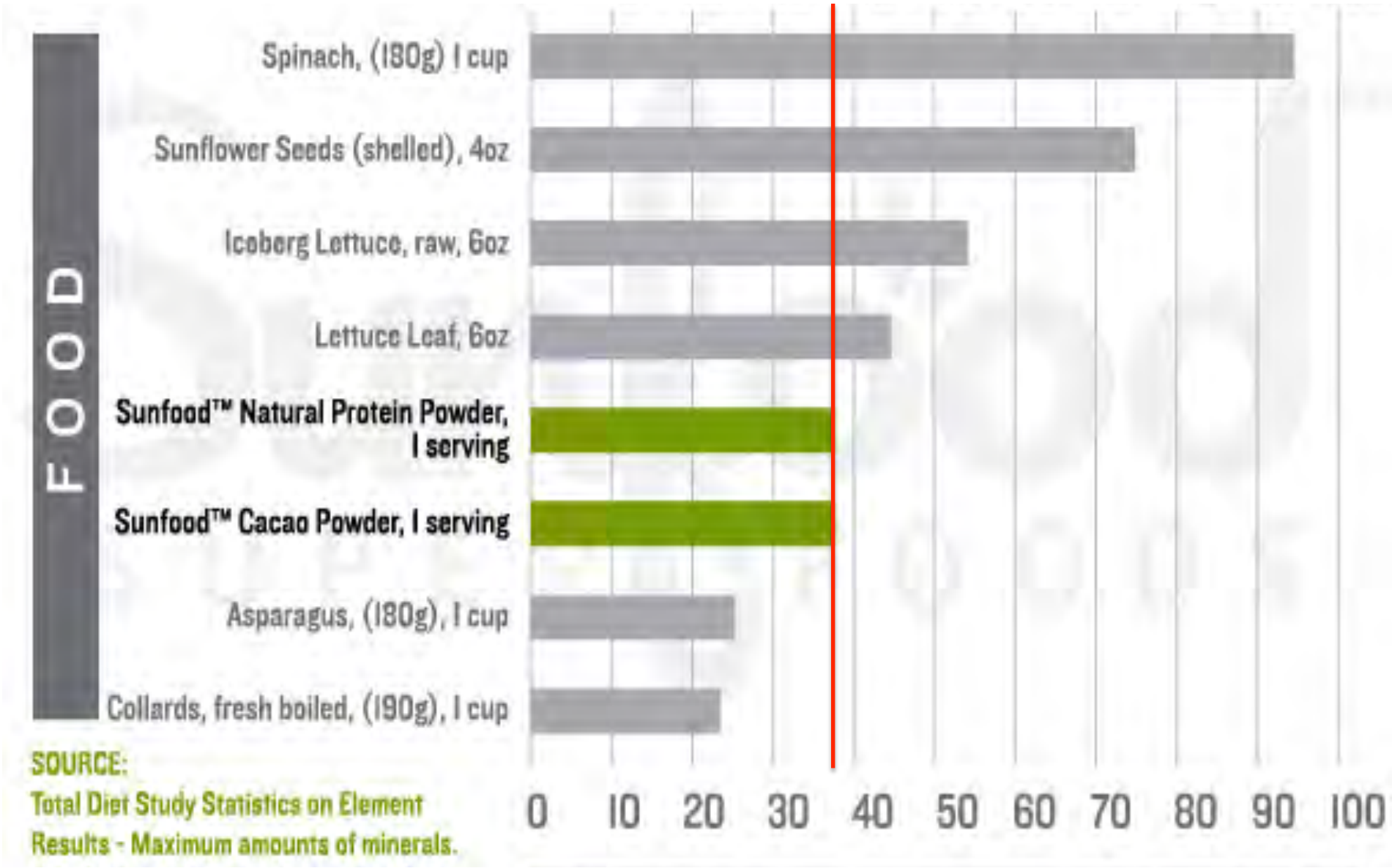
Cadmium

- Cadmium (silvery gray) is widely distributed in the environment, due to extensive industrial use
- Sewage sludge, which is used as fertilizer and soil conditioner, is an important source of soil pollution with Cadmium

Cadmium in Food in ug/100 g

FOODS	1972–1973		1973–1974		1974–1975		1975	1976
	Range	Average[a]	Range	Average[a]	Range	Average[a]	Range	Average[a]
Dairy products	1–6	trace (5/30)	1–14	1 (4/30)	trace	trace (4/20)	1–2	0.2 (3/20)
Meat, fish, and poultry	1–6	1 (12/30)	1–6	2 (21/30)	trace	trace (11/20)	1–3	1.0 (17/20)
Grains and cereals	2–5	1 (30/30)	2–5	3 (29/30)	5–8	trace (19/20)	2–5	3.0 (20/20)
Potatoes	2–12	5 (30/30)	2–13	5 (29/30)	5–12	4 (20/20)	2–9	5.0 (20/20)
Leafy vegetables	1–28	5 (30/30)	1–14	4 (28/30)	5–14	5 (20/20)	2–10	4.0 (19/20)
Legumes	1–3	trace (10/30)	1–10	1 (8/30)	trace	trace (3/30)	1–7	1.0 (14/20)
Root vegetables	1–6	2 (24/24)	1–31	3 (24/30)	trace	trace (16/20)	1–8	2.7 (19/20)
Garden fruits	1–6	2 (25/25)	1–10	2 (23/30)	trace	trace (17/10)	1–4	2.0 (18/20)
Other fruits	1–2	trace (4/30)	1–6	trace (3/30)	trace	trace (5/20)	1–2	0.3 (5/20)
Oils, fats, shortening	1–6	3 (29/30)	1–7	2 (24/30)	trace	trace (17/20)	1–3	1.6 (18/20)
Sugars and adjuncts	1–6	1 (13/30)	1–9	1 (12/30)	trace	trace (8/20)	1–3	1.1 (14/20)
Beverages	1–8	trace (5/30)	1–3	trace (6/30)	trace	trace (1/20)	0–1	0.2 (3/20)

Main Cd Levels in Food



Cadmium

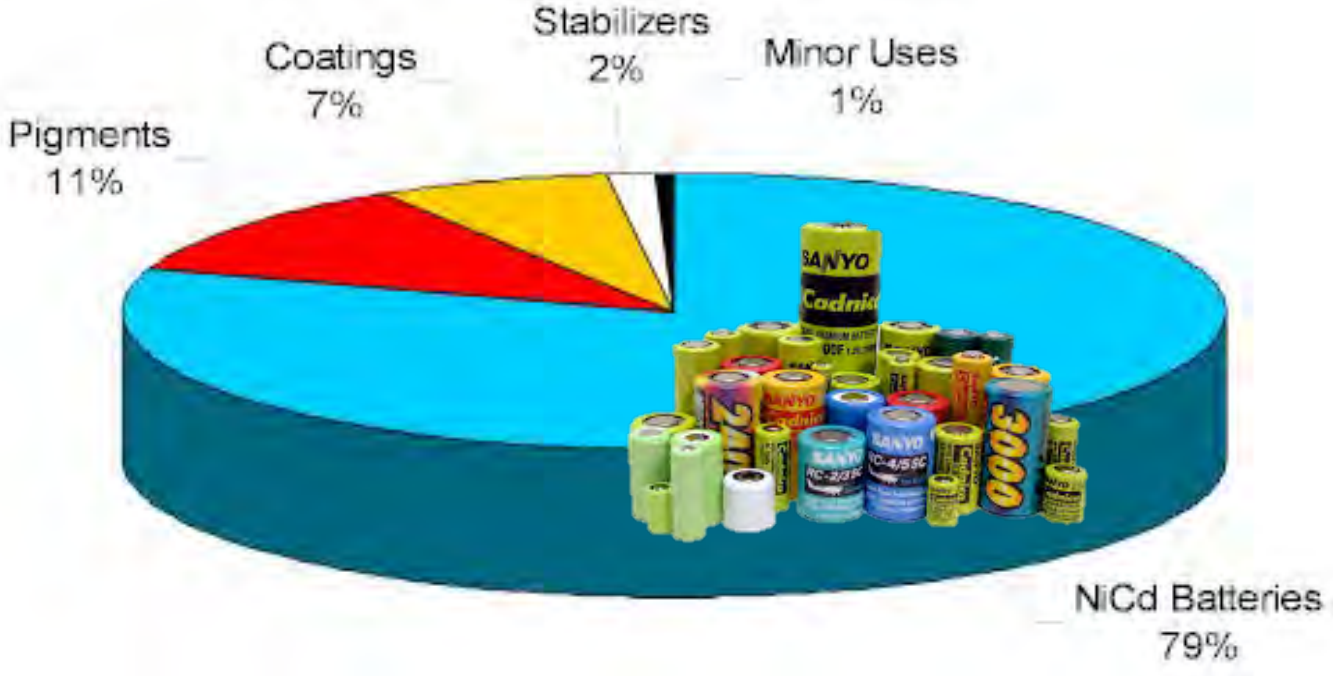
- **Cadmium accumulates in the human body, especially in the liver and kidney**

- **Toxicity→ reduced body height and multiple fractures, hypertension**

- **Case: Chronic cadmium intoxication in humans occurred in Japan after the consumption of rice heavily contaminated as a result of environmental pollution 0.1 to 1 mg/day**

Cadmium application

Application: battery, stabilizer (PVC), coating (aluminium), electronic compound, fossil fuel, fertilizer, cadmium pigments-bright yellow (plastic, ceramic, glass)



Regulation

- **FDA: 3.7 to 14.4 µg/day, TWI is 0.4 to 0.5 mg drinking water and bottled water 0.005 mg/l**
- **For foods, no limit values have been set**
- **In Europe for meat the limit is 0.05 ppm, in some fishes and in cereals is 0.1 ppm while in spices and vegetables is from 0.05 to 0.2 ppm reg. EU 1881/2006**

Toxic Phenolic substances

- Contribute to the bitter taste, flavor, color, etc.
- Phenolic acids: flavonoid, lignin, gallic acid, tannins
- Highly toxic phenolic substances: coumarin, safrole, phenolic amines (gossypol, catecholamines), myristicin

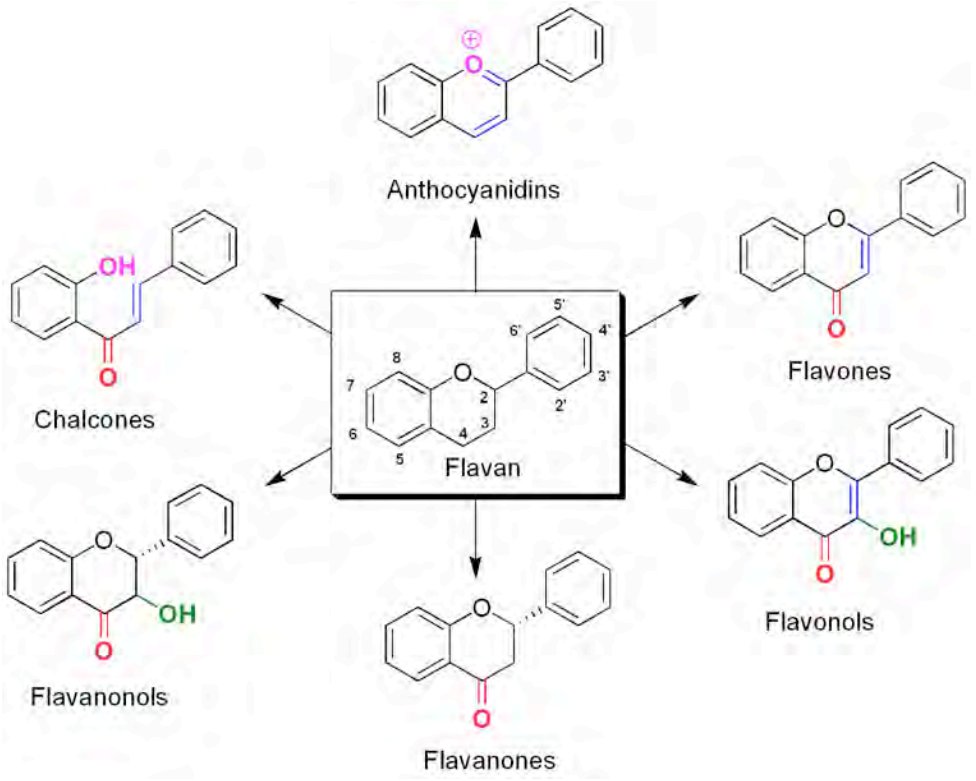
Flavonoids

Plant pigments that are widely present in human food (most are present as β-glucosides)

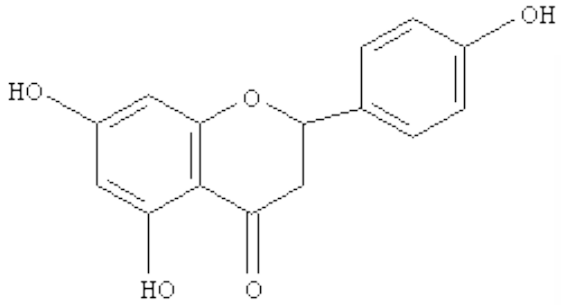
More than 1 g ingested daily in the diets

Divided into 6 groups

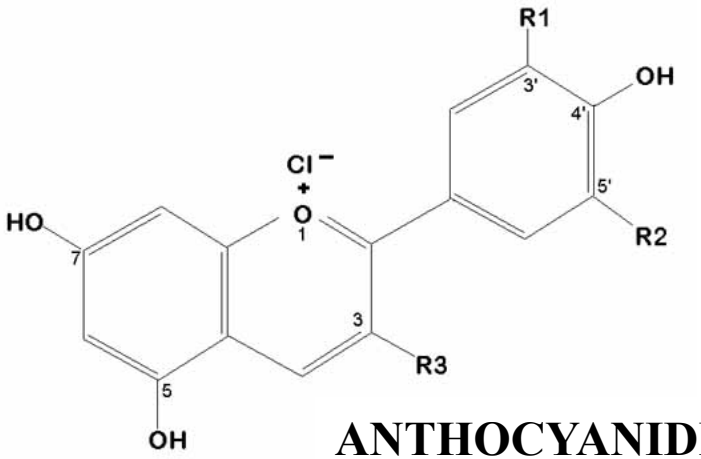
- **Flavanone**
- **Flavone**
- **Anthocyanidin**
- **Isoflavone**
- **Chalcone**
- **Aurone**



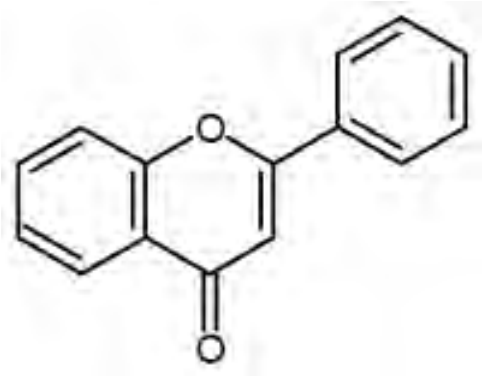
Flavonoids Chemical Sketch



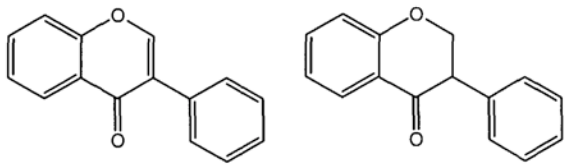
FLAVANONE



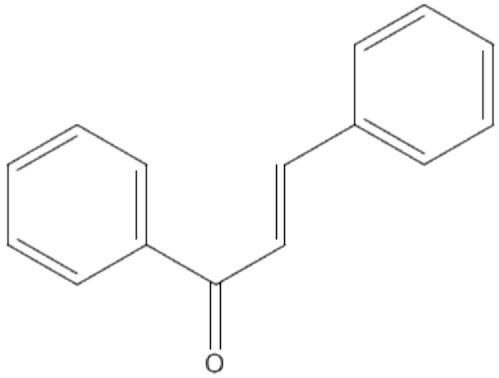
ANTHOCYANIDINE



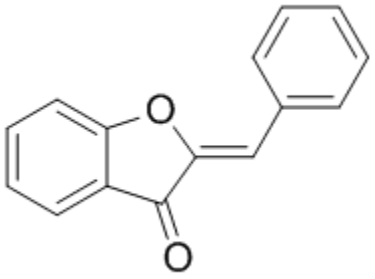
FLAVONE



ISOFLAVANONE



CHALCONE



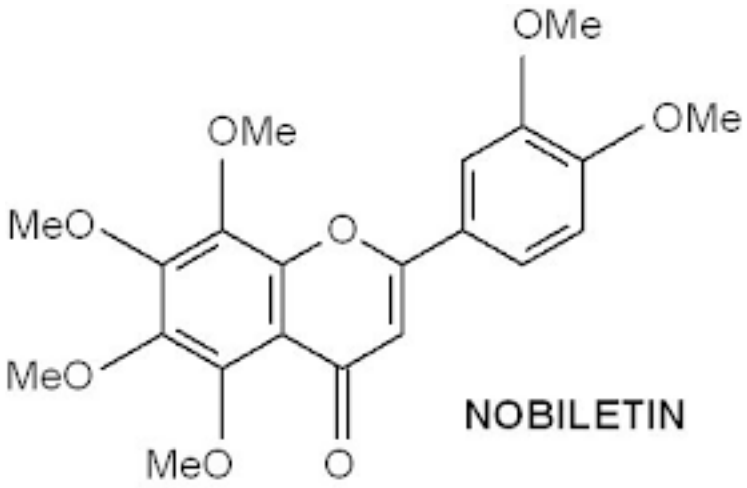
AURONE

Natural Source of Flavonoids

- **Sources of flavonoids include:**

apples, apricots, blueberries, raspberries, strawberries, pears, black beans, cabbage, onions and tomatoes

- **Fruit peel**
  - **oily orange peel: 2 mg nobitelin/100 ml oil**
    0.3 mg tangeretin/100 ml oil



NOBILETIN

Toxicity of Flavonoids

- In very high amounts (for ex., 140 grams per day), flavonoids do not appear to cause unwanted side effects
- When raised to the level of 10% of total caloric intake, flavonoid supplementation has been shown NON-TOXIC
- Poor intake of fruits and vegetables - or routine intake of high-processed fruits and vegetables - are common contributing factors to flavonoid deficiency
- Toxicity: carcinogenic (quercetin in cereal crops)

Toxicity of Flavonoids

At low concentrations → The flavonoids are potentially anticarcinogenic because they can block and inhibit the excessive cell division characterized by cancer

In fact, they can inhibit enzymes, such as protein kinases, that are involved in cellular proliferation and tumor progression

Flavonoids can be considered *anticarcinogens*

At high concentration of flavonoids they may promote cancer formation because they damage the chromosomes and DNA in cells, leaving them more susceptible to cancer promotion

Toxicity of Flavonoids

- **However, thay can inhibit some enzymes that can alter normal body functions and metabolisms**
- **They can interfere with the metabolism of drugs and with mineral absorption in our bodies**
- **Daily intake: 150-250 mg/day**
- **The FDA has <u>not yet established recommended daily intake levels for flavonoids</u>**

"Just because something comes from a natural source doesn't mean it can't hurt you"

Tannins

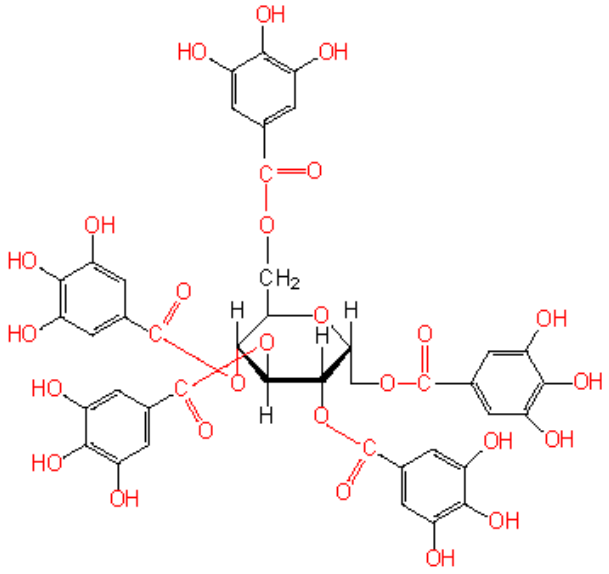
TANNINS

- **Hydrolysable Tannins**
  - **Gallotannins**
  - **Ellagitannins**
- **Complex Tannins**
- **Condensed Tannins**

Tannins are phenolic plant secondary compounds and are widely distributed through the plant kingdom, especially legumes which affect animal performance in many countries

Tannins

- **Two subfamily of tannins (polyhydric phenols) can be distinguished on the basis of degradation behavior and botanical distribution, namely *hydrolyzable tannins* and *condensed tannins***
- **The *hydrolyzable tannins* are tannic acid, also known as gallotannic acid, gallotannin, or simply tannin**
- **The *condensed tannins* are well known as Flavonoids**
- **Toxicity: cause acute liver injury, i.e. liver necrosis and fatty liver**



Source of Tannins

- **Fruits, tea (highest content), coffee, cocoa, grape, wine**
- **A cup of ground coffee: 72-104 mg**
- **Instant coffee: 11-128 mg**

1 g ingest of Tannins per day




Toxicity of Tannins

- If ingested in excessive quantities they inhibit the absorption of minerals such as iron and lead anemia because they are metal ion chelators
- Tannins have been shown to precipitate proteins which inhibits in some ruminant animals the absorption of nutrients from high-tannin grains such as sorghum
- Tannic acid does not affect absorption of other trace minerals such as zinc, copper and manganese in rats
- In sensitive individuals, a large intake of tannins may cause bowel irritation, kidney irritation, liver damage, irritation of the stomach and gastrointestinal pain

Cyanogenic Glycosides

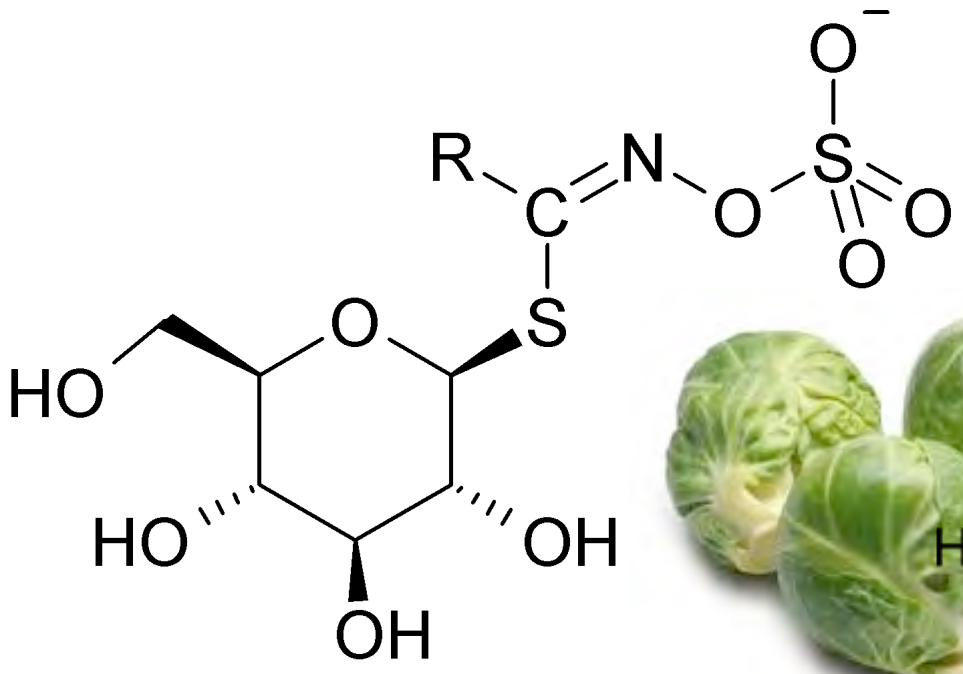
- Glycosides from cyanide formed by enzymatic hydrolysis
- Sources: plants
- Lethal intakes by humans: 0.5-3.5 mg/kg body weight
- Beans: 200-300 mg/100 g in selected breeding of low-cyanide varieties of beans
- Cassava: 1-60 mg/100 g in fermented cassava

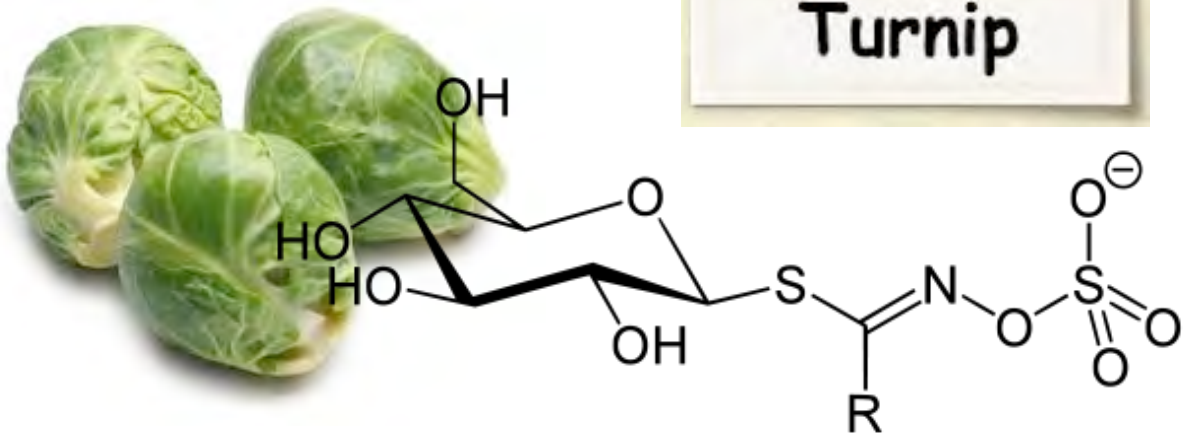
Hydrogen Cyanide contents of some foodstuffs

Food	HCN (mg/100 g)
• Lima Beans	210–310
• Almonds	250
• Sorghum sp.	250
• Cassava	110
• Peas	2.3
• Beans	2.0
• Chick peas	0.8

Glucosinolates

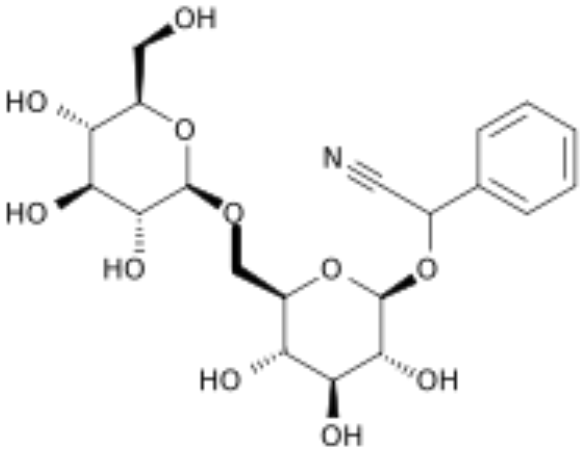
- **Substances that can be considered as natural toxins, but also as antinutrients**
- **Source: cabbage and turnips**
- **Toxicity: cytotoxic and mutagenic**

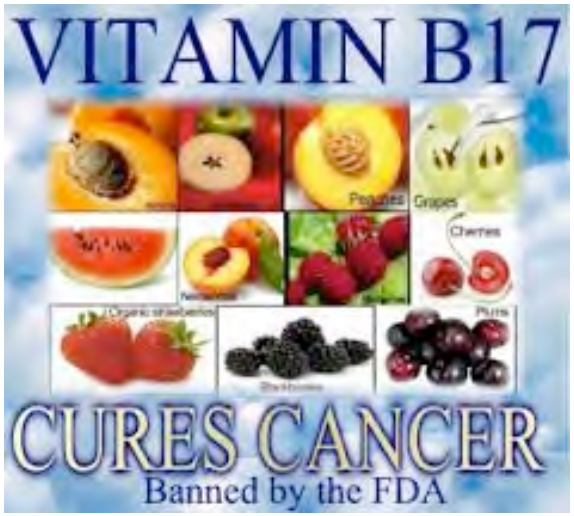




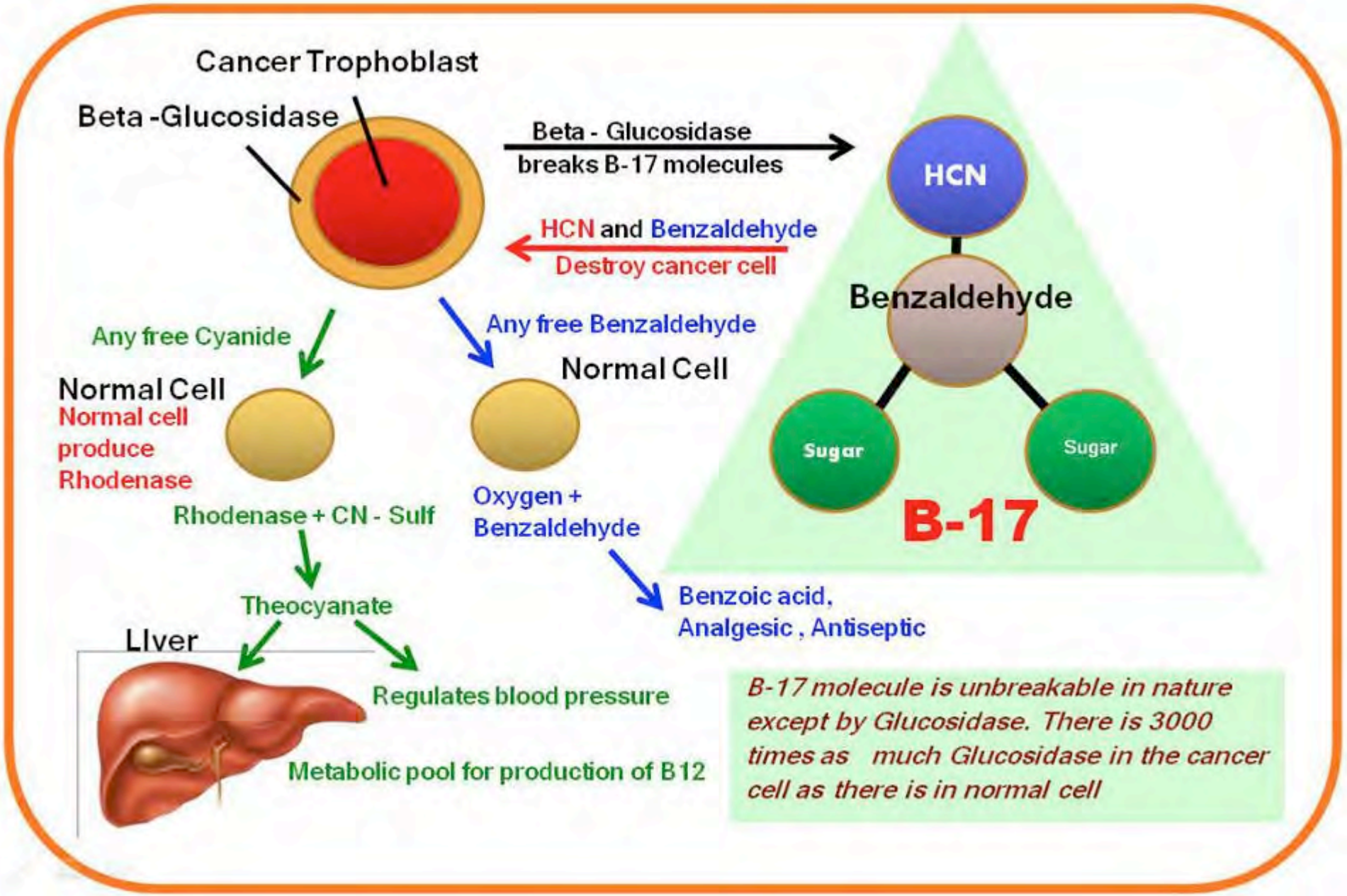
Laetrile or Vitamin B17

Laetrile is actually used only in Mexico as anticancer drug. It is based on its HCN production and interference on cell metabolism





Laetrile or Vitamin B17



Biogenic Amines

- **Natural toxins → plant as well as of microbial origin**

- **Source: Fruit (avocado, banana, orange, tomato, potato, pineapple)**

- **Type: dopamine, epinephrine, norepinephrine, serotonin, tyramine**

Toxic Microbial Metabolites

Biogenic amines (biogenic substance with an amine group)

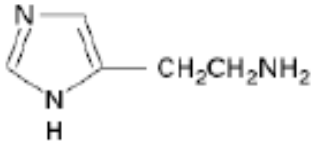
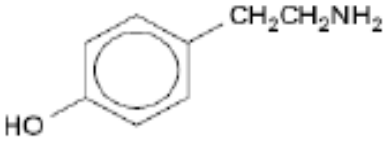
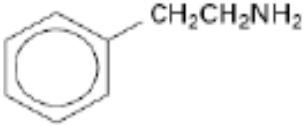
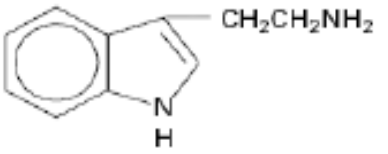
- **The main producers of biogenic amines in foods are Enterobacteriaceae and Enterococci (Enterobacteriaceae → cadaverine formation while lactobacilli → tyramine formation)**
- ***Toxicity and symptoms*** **The symptoms of intoxication, persisting for several hours, include burning throat, headache, nausea, hypertension, numbness and tingling of the lips and vomiting**

Toxic Microbial Metabolites

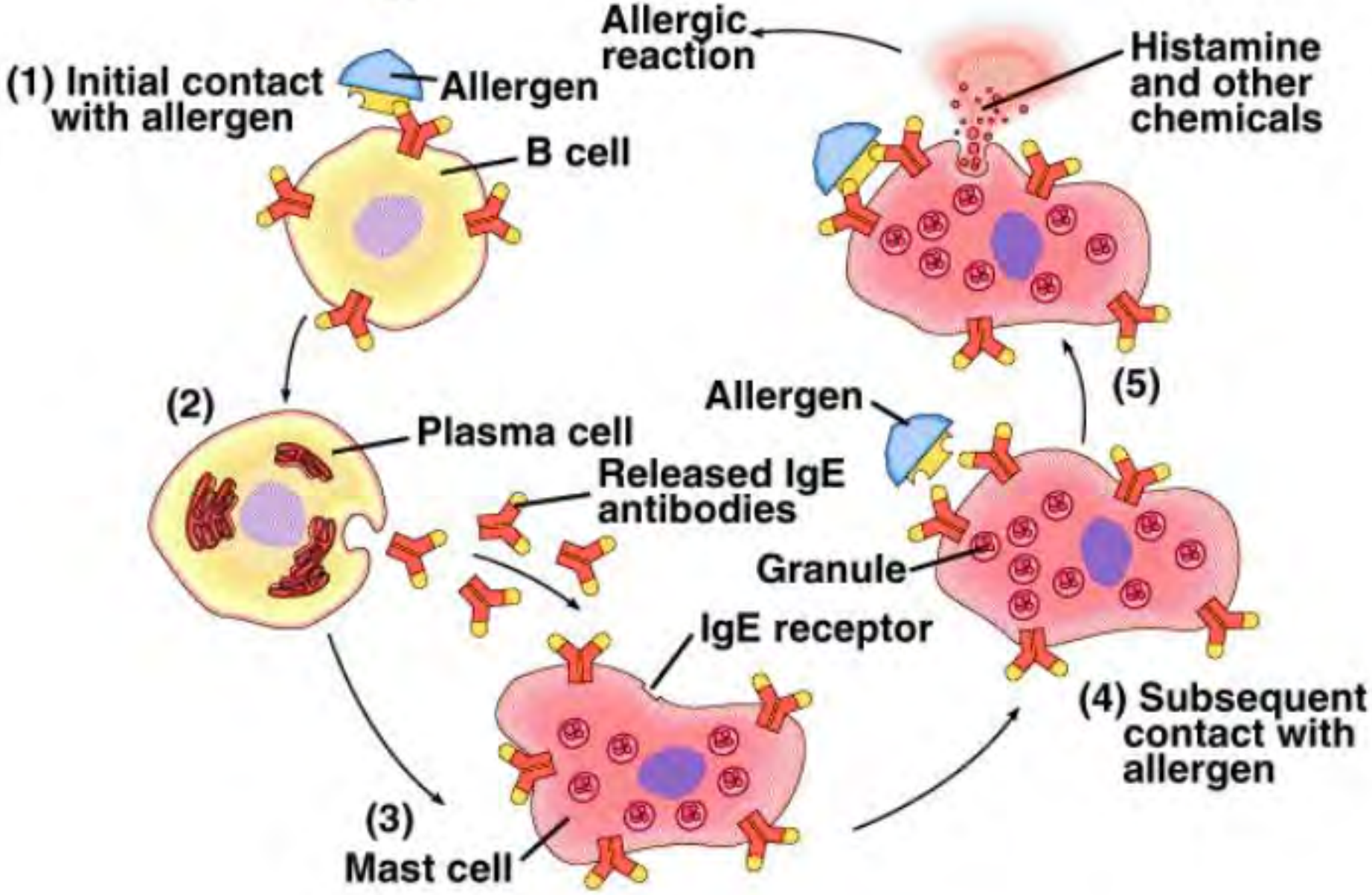
Biogenic amines (biogenic substance with an amine group)

- ***Type of food involved*** **associated with lactic fermented products, particularly wine, cheese, fish, and meat, fruits, vegetable**
- ***Environmental condition:*** **amino acid precursor, low pH of the product, high NaCl concentrations, microbial decarboxylase activity. For instance, fresh fish (mackerel, tuna) contain high levels of histidine which is readily decarboxylated to histamine by Gram-negative bacteria, e.g., *Proteus morganii***
- **Prevention: Pasteurization of cheese milk, good hygienic practice and selection of starters with low decarboxylase activity**

Toxic Microbial Metabolites

Amine	Formula	Precursor
Ethylamine C_2H_7N	$CH_3CH_2NH_2$	Alanine
Putrescine $C_4H_{12}N_2$	$H_2N(CH_2)_4NH_2$	Ornithine
Histamine $C_5H_9N_3$		Histidine
Cadaverine $C_5H_{14}N_2$	$H_2N(CH_2)_5NH_2$	Lysine
Tyramine $C_8H_{11}NO$		Tyrosine
Phenylethylamine $C_8H_{11}N$		Phenylalanine
Tryptamine $C_{10}H_{12}N_2$		Tryptophan

Allergic Reaction due to Amines



safe	accepted	hazardous
100 ppm	1000 ppm	>2000 ppm

beer

wines

sausages

gouda cheese

brie / camembert

blue cheese / gorgonzola

terasi (fish paste)

yoghurt

sauerkraut

Central Stimulants

- Increase the activity of nervous system
- Methylxanthines: caffeine, theophylline, theobromine
- Caffeine: coffee (1 cup = 150 mg), tea, cocoa, cola (0.1 - 0.15 mg/mL)

Natural contaminants

Microbial toxin

- The existence of microorganism is related to food environment (water, pH, temperature, oxygen, etc.)
- Microorganisms degrade the food components enzymatically and excrete their metabolites
- The resulting is the loss of food structure or formation of off-smells is regarded as spoilage

Microbial Toxin

Bacterial toxin can be classified:

1. **sub unit toxin (*Clostridium botulinum*)**
2. **membrane-affecting toxin (*S. aureus*)**
3. **lesion-causing toxins (*C. perfringens*, *B. cereus*)**
4. **immuno-active endotoxins (Gram negative bacteria toxin)**

Microbial Toxin by *Clostridium*

Characteristic of microbial toxin:

- **Type of toxin (*C. botulinum*): A, B, C1, C2, D, E, F and G**
  (Type A → is the most lethal toxin)
- **Type A, B, E & F are toxic to humans**
- **Symptom (after 12-72 h): nausea, vomiting, headache, double vision, paralysis, respiratory problem**
- **Mortality is in the range 30-65%**
- **Stability: heat sensitive, 80 ^{o}C for 10 min in addition are acid resistant and survives the gastric passages**
- **Environmental condition: *C. botulinum* grows at pH>4.6, 37^{o}C**
- **Type of food: meat, fish, food with low-neutral pH (> 4.60)**
- **Prevention: addition of nitrite, low pH, low aw, addition of salt, through heating, refrigerated storage**

Characteristic of Toxin by *S. aureus*

- Type of toxin: A, B, C1, C2, C3, D and E
- Toxicity: 1-25 mg toxin are able to make sickness
- Symptom (30'- 6 h): vomiting, diarrhea (dehydration)
- Stability: heat resistant (100 ^{o}C for 1 h)
- Environmental conditions: 7-46 ^{o}C (opt. 37 ^{o}C), pH 4-9 (opt. pH 7), aw > 0.86, NaCl up to 15%
- Toxin production: >12 ^{o}C, aw 0.9, pH > 4.6, aerobic condition
- Type of food: dairy cream, ice cream, cured meat (sausages), canned food
- Prevention: proper storage (refrigerated), personal hygiene

Characteristic of Toxin *C. perfringens, B. cereus*

- **Type of toxin: A, B, C, D , E, F**
- **Toxicity: > 10^8 cell to release toxin**
- **Symptom (after 8 - 24 h): cramps, diarrhea**
- **Mortality is very low (range 3-4%)**
- **Stability: heat sensitive (0.3 min, 100 ^oC), are heat stable for 17.6 min at 100^oC**
- **Environmental conditions: 15-50 ^oC (opt. 40 ^oC), pH 5-8 (opt. pH 7), aw > 0.93**
- **Type of food: meat, canned foods (improper sterilized)**
- **Prevention: proper storage (refrigerated, < 7 ^oC), personal hygiene, heating > 65 ^oC**

Food-borne bacterial pathogens and associated diseases

Organism	Pathogenicity	Incubation time (hours)	Duration of disease (days)
Salmonella	infection	6–36	1–7
Shigella	infection	6–12	2–3
Escherichia coli	infection	12–72	1–7
Yersinia enterocolitica	infection	24–36	3–5
Campylobacter jejuni	infection	3–5 (days)	5–7
Listeria monocytogenes	infection	variable	—[a]
Vibrio parahemolyticus	infection	2–48	2–5
Aeromonas hydrophila	infection	2–48	2–7
Staphylococcus aureus	toxin in food	2–6	≤1
Clostridium botulinum	toxin in food	12–96	1–8[b]
Clostridium perfringens	toxin in intestine	8–22	1–2
Bacillus cereus[c]	toxin in food	1–5	≤1
Bacillus cereus[d]	toxin in intestine	8–16	>1

[a] Affects people with a predisposing factor; high mortality rate.

[b] High mortality rate; complete convalescence takes 6–8 months.

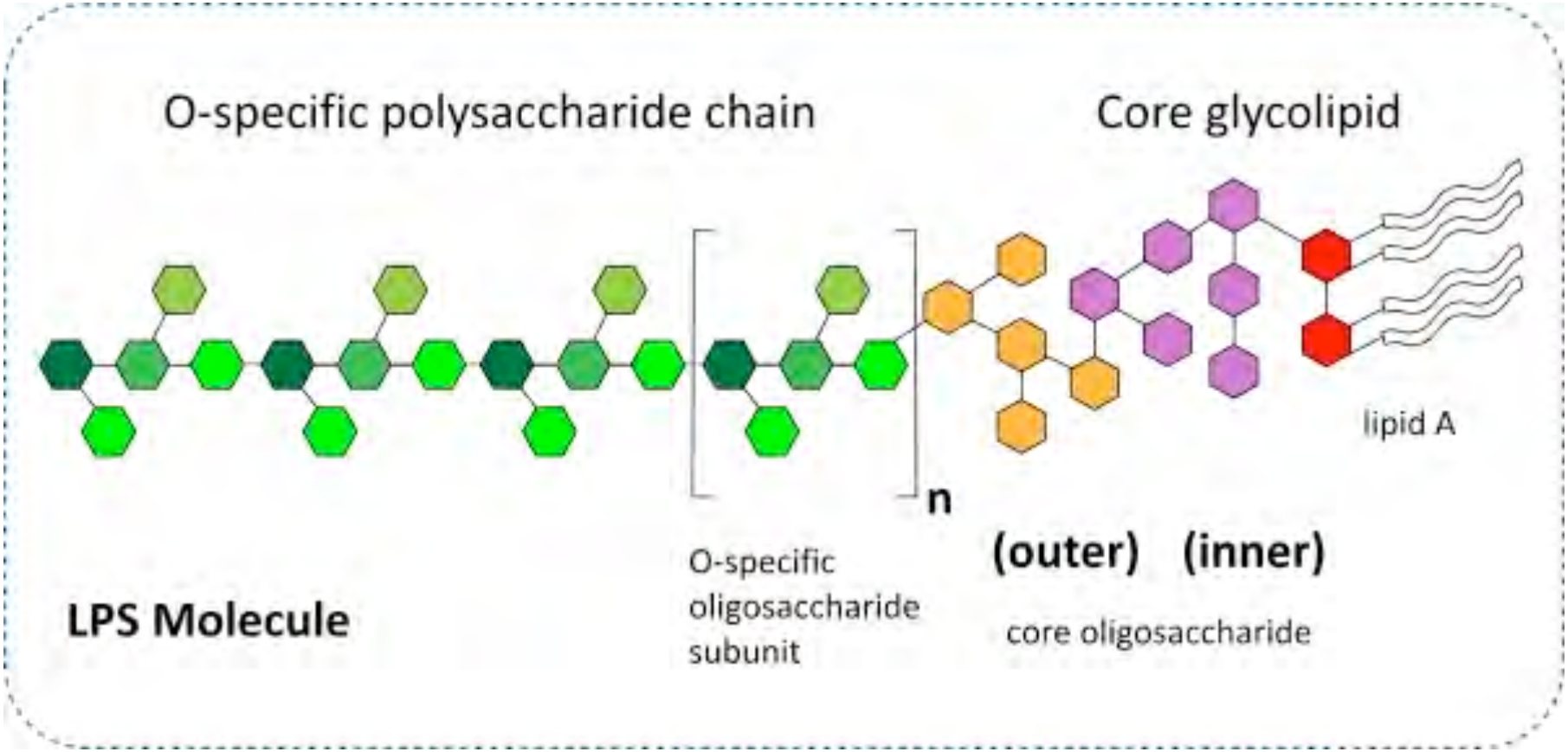
[c] Emetic type.

[d] Diarrheal type.

Characteristic of Toxin by Gram -

- **Lipopolysaccharide (LPS) are found in cell wall of Gram (-) bacteria**
- **Toxicity: causing inflammation**
- **Symptom: fever, painful joints, shock, death**
- **Stability: are heat resistant**
- **Environmental conditions: 15-40 ^{o}C pH 4.5 aw > 0.99**
- **Type of food: any type of food**
- **Prevention: proper storage, personal hygiene, avoid cross contamination (between raw food and cooked food)**

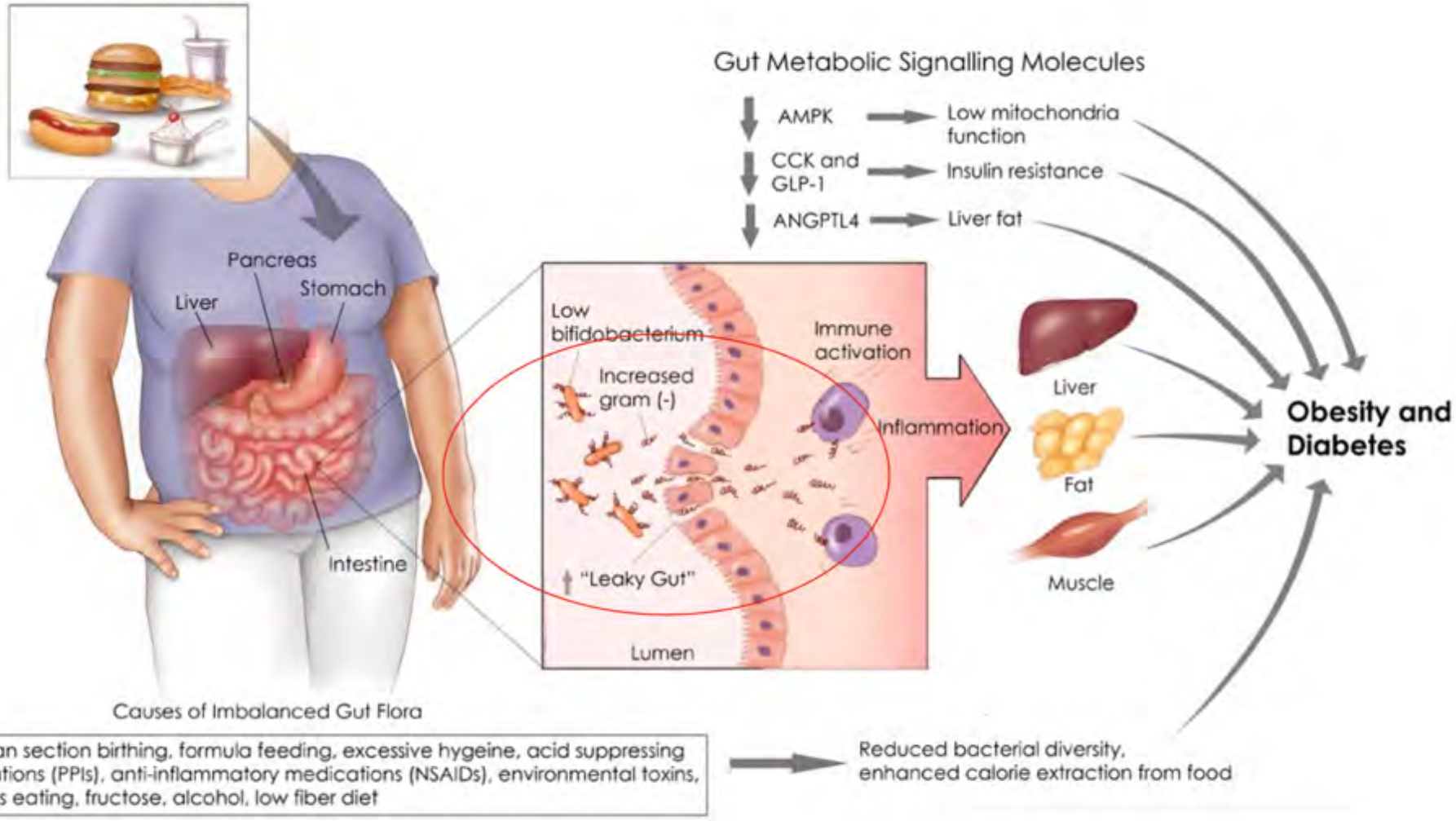
Structure of Endotoxin



Toxicity is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components

The cell wall antigens (O antigens) of Gram-negative bacteria are components of LPS

What is Endotoxin

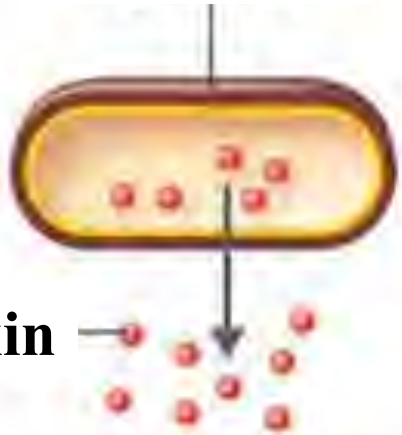


Characteristic of Endotoxin and Exotoxin

Property	Endotoxin	Exotoxin
Chemical nature	Polysaccharide (10kDa)	Protein (50-100kDa)
Relationship to cell	Part of outer membrane	Extracellular
Denatured by boiling	No	Usually
Potency	Low (> 100 mg)	High (1 mg)

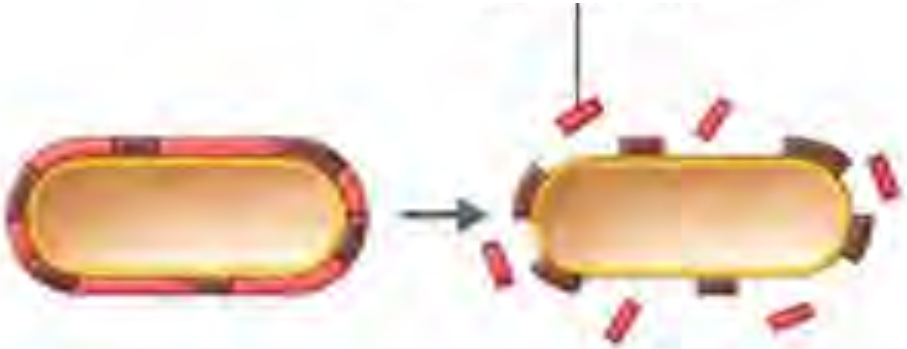
Differences Between Endotoxin and Exotoxin

Cell wall



(a) Exotoxins are proteins produced inside pathogenic bacteria, most commonly gram-positive bacteria, as part of their growth and metabolism. The exotoxins are then secreted or released into the surrounding medium following lysis.

Endotoxin

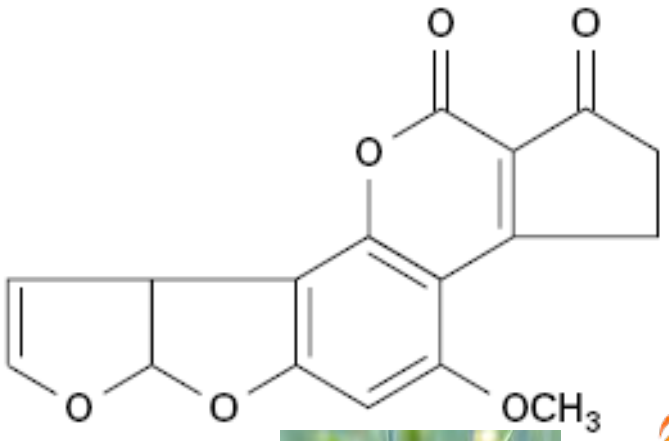


(b) Endotoxins are the lipid portions of lipopolysaccharides (LPSs) that are part of the outer membrane of the cell wall of gram-negative bacteria (lipid A; see Figure 4.13c). The endotoxins are liberated when the bacteria die and the cell wall breaks apart.

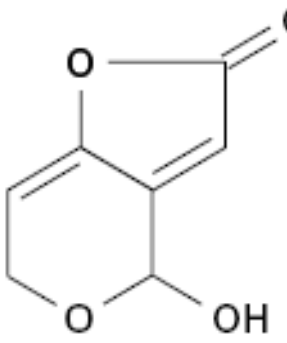
Mycotoxin

- **Mycotoxins are secondary metabolites of fungi (*Aspergillus, Penicillium, and Fusarium) w*hich can induce acute as well as chronic toxic effects (i.e., carcinogenicity, mutagenicity) in animals and human**
- **Toxic syndromes resulting from the intake of mycotoxins by man and animals are known as mycotoxicoses "*Yellowed Rice Disease*" in Japan caused by *Penicillium spp***
- **Stability: are stable and very resistant to cooking**
- **The absence of viable molds in foods does not necessarily mean there are no mycotoxin**
- **Crops affected by mycotoxin: cereal, spices, soybean, peanut**

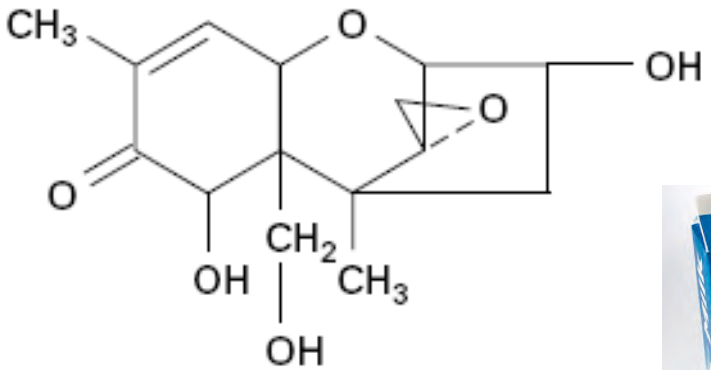
Some Mycotoxin Structures



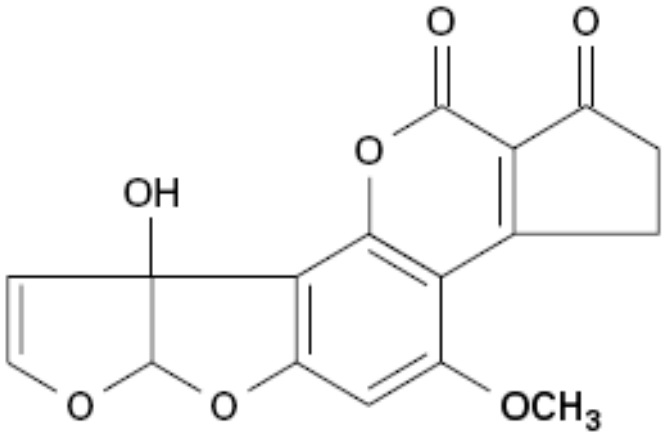
Aflatoxin B1



Patulin



Deoxynivalenol



Aflatoxin M 1

Mechanisms of Action of Mycotoxins

- It's impossible to define a single mechanism for each mycotoxin
- This variety of teratogenic effects is related to their heterogeneous origin and chemical nature
- The effect on the fetus can occur at any one of the developmental stages (from gametogenesis to weaning period)
- Data describing prenatal effects of mycotoxins are poor, incidental and in some cases were only allusions to the morphopathologic aspects

Mode of Action of Aflatoxins (AF)

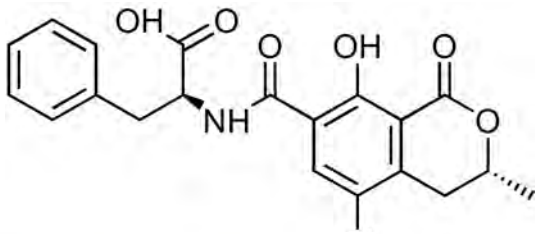
- AF have been reported in literature to be embryotoxic and teratogenic
- Early embryos being more susceptible than older embryos
- High doses later stages = Low doses early stages
- AFB1 injected in hamster at 4 ppm is teratogenic

- Increasing of AFB1 from 4 to 6 ppm increase only the percentages of abnormalities
- Preincubation of AFB1 with DNA prior injection reduces teratogenic damages and liver damages
- AFB1-DNA adducts doesn't pass the placenta

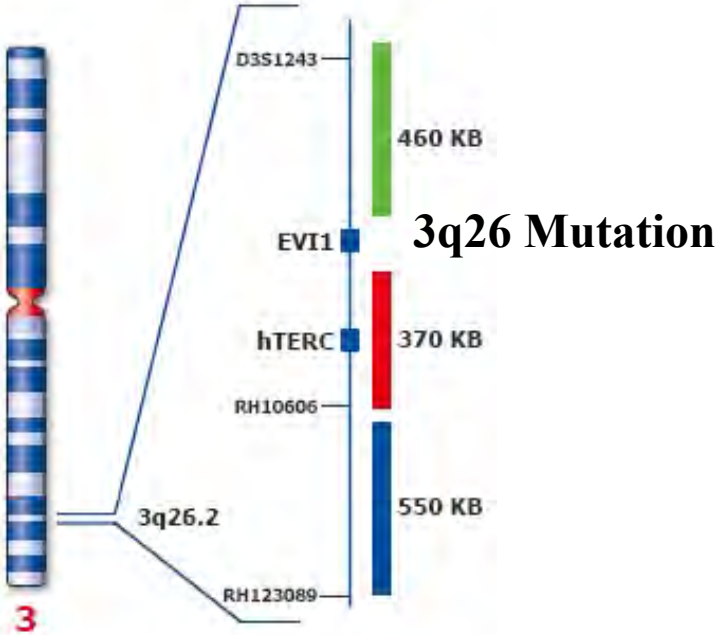
Ochratoxin A

In the 1950s, in Bulgaria, Yugoslavia and Romania was described a kidney disease occurring in these Balkan countries and restricted to individuals from farming households

(R)-N- [(5-Chloro- 3,4-dihydro- 8-hydroxy- 3-methyl- 1-oxo- 1H-2-benzopyran-7-yl) -carbonyl]- L-phenylalanine



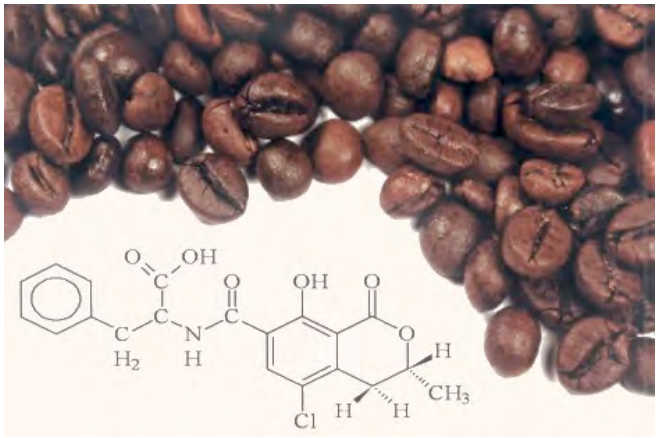
In 1964, the WHO reviewed critically all the available data and provided the following description of the disease: "*…progressive and very gradually developing renal failure with insidious onset. It develops without a nephrotic syndrome and usually without hypertension*
There is a marked anaemia, mild proteinuria, and trivial urinary deposit"



Balkan Endemic Nephropathy (BEN)

The clustering of BEN supports that a genetic predisposition may be involved.
Toncheva in 1998, suggest that a genetic factor for BEN could be due to genes located in a region between 3q25 and 3q26

Ochratoxin A

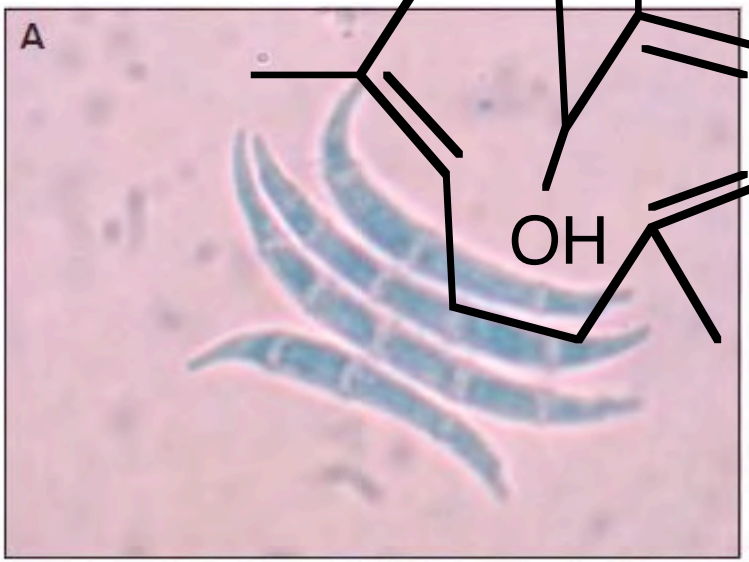


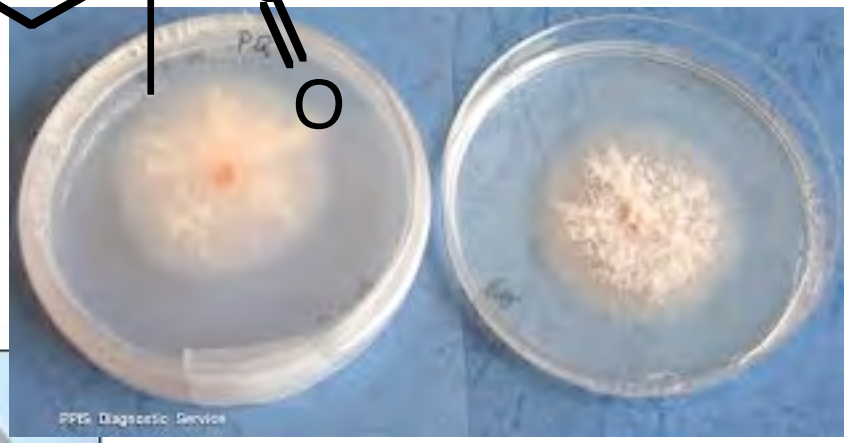
Wine, beer, chocolate, coffee, cereals, etc.

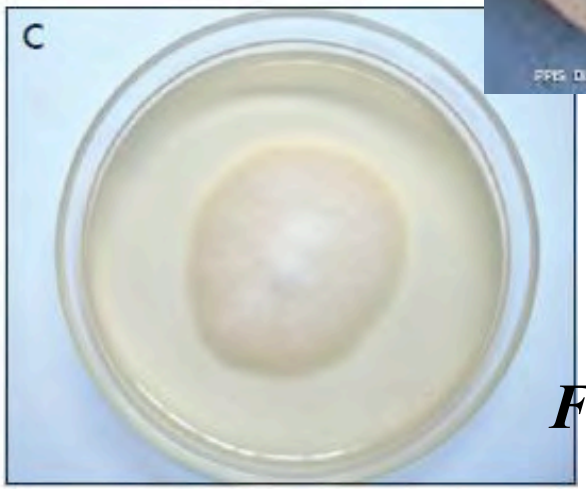
Mode of Action of Ochratoxin A (OTA)

- **OTA is involved in natural outbreaks of swine and cattle abortion**
- **OTA administered to pregnant rats on 10 day of gestation induced a high percentage of fetal death and embryonic resorption**
- **OTA passes the placenta and may accumulate in the muscles, liver and kidneys**
- **OTA leds a similar syndrome produced by hypervitaminosis A and leds facial clefts, exencephaly, cranio-facial malformations**

Fusaproliferin p.m. 444



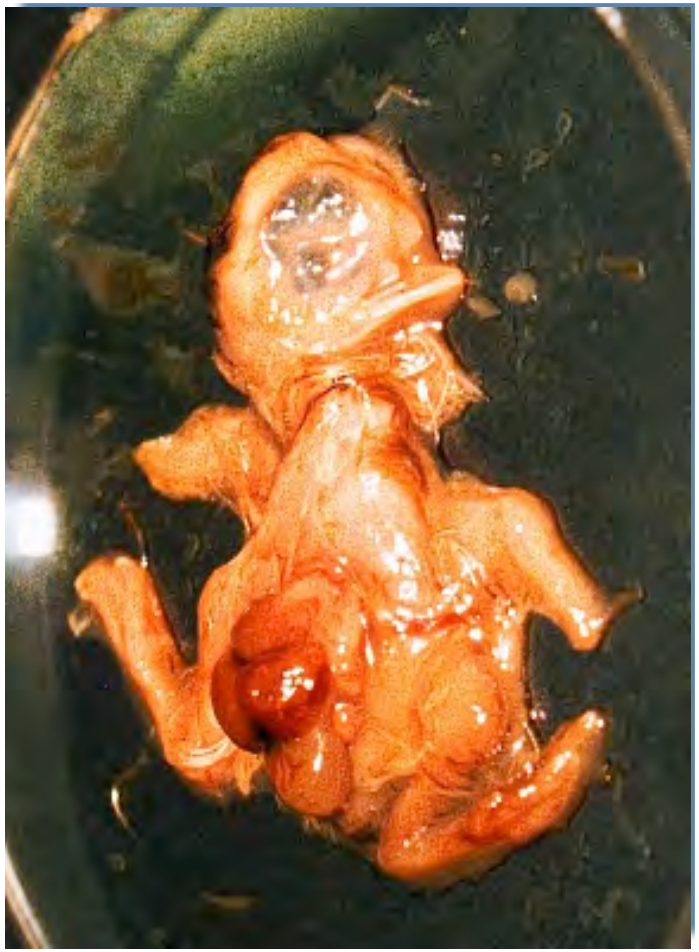




Fusarium proliferatum

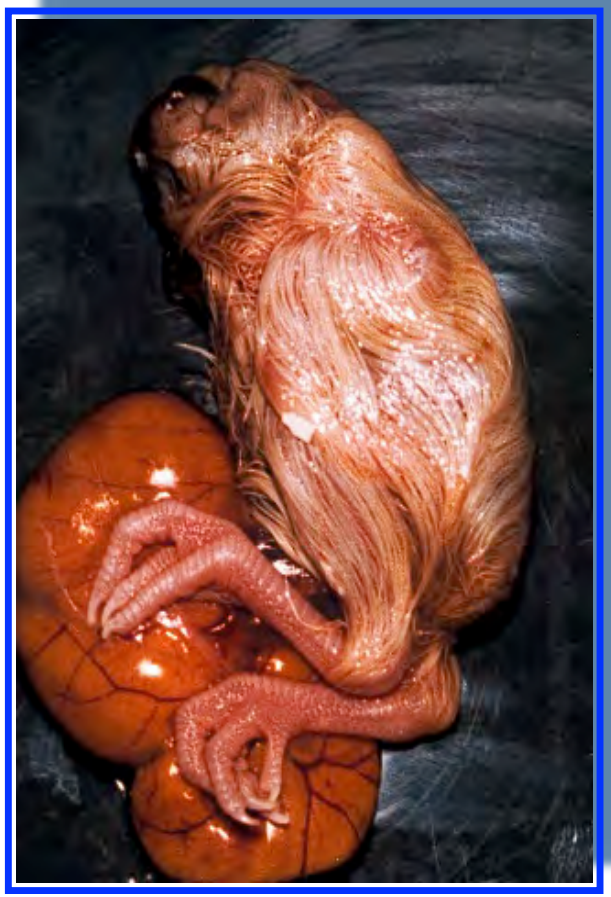
World-wide occurrence of Fusaproliferin

Teratogenic effects of Fusaproliferin

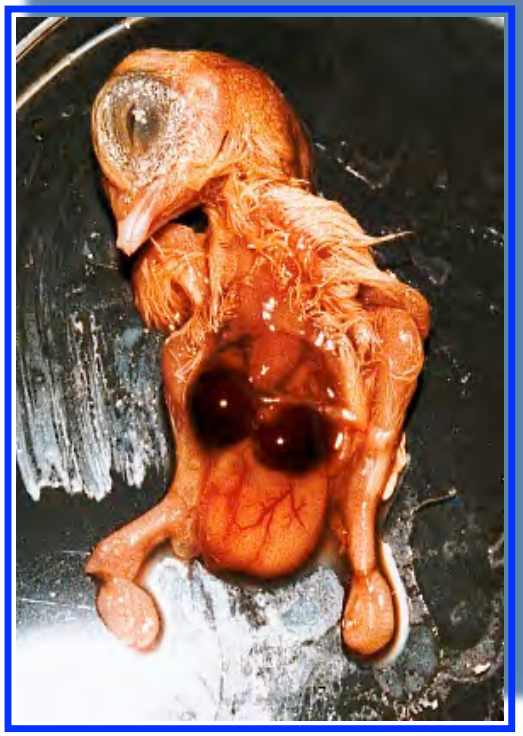


Abnormal development of body

Teratogenic effects of Fusaproliferin



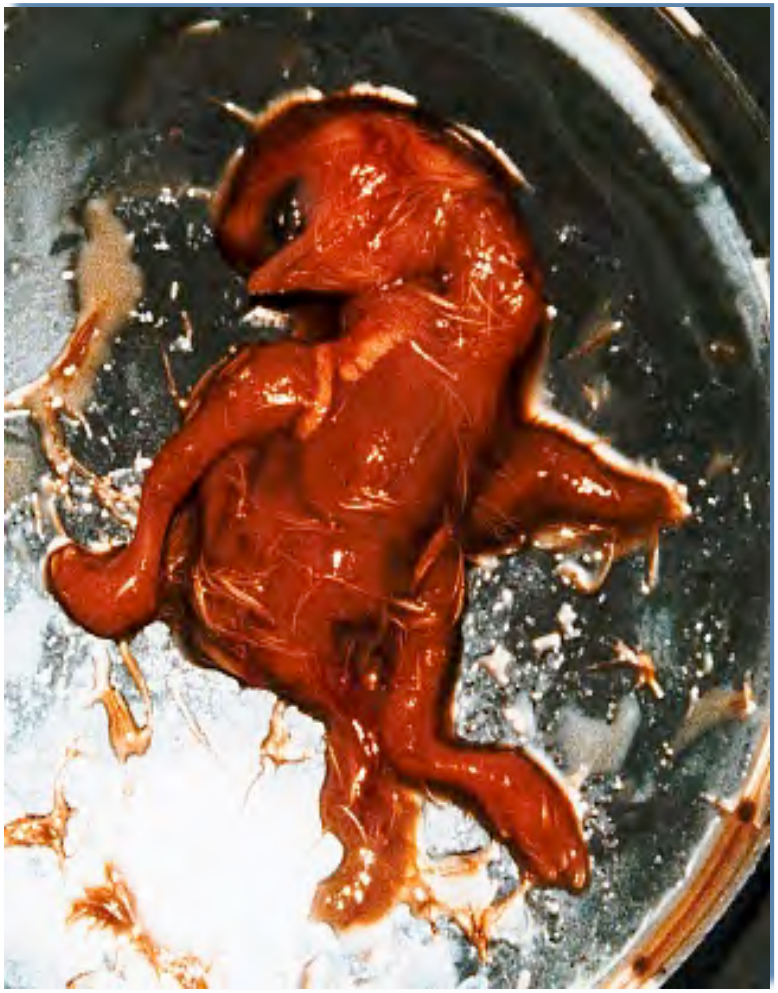
Absence of Head



Abnormal development of the abdomen region

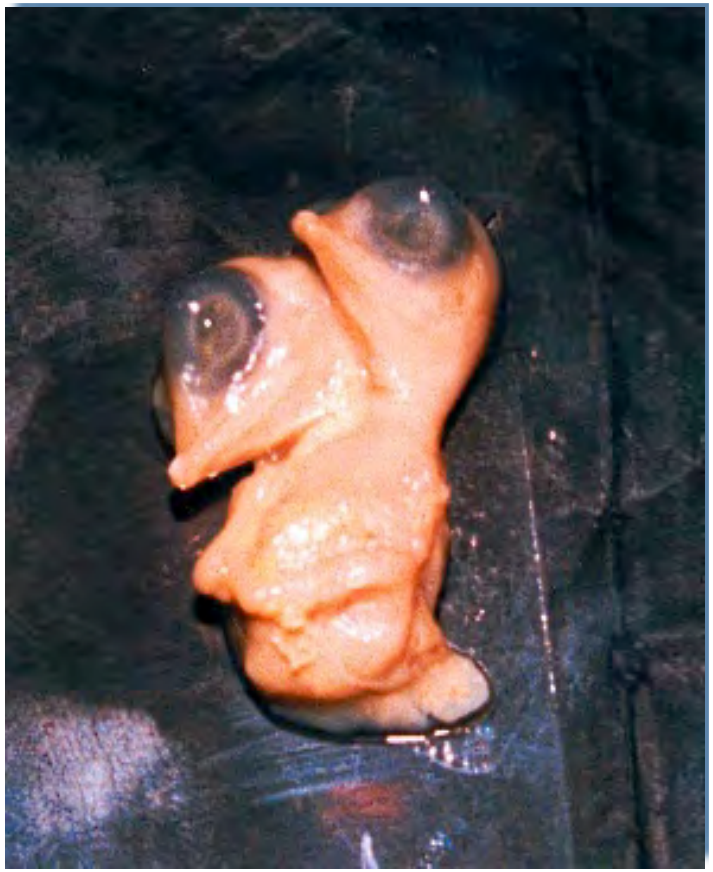
Teratogenic effects of Fusaproliferin

Incomplete closure of the umbilicus

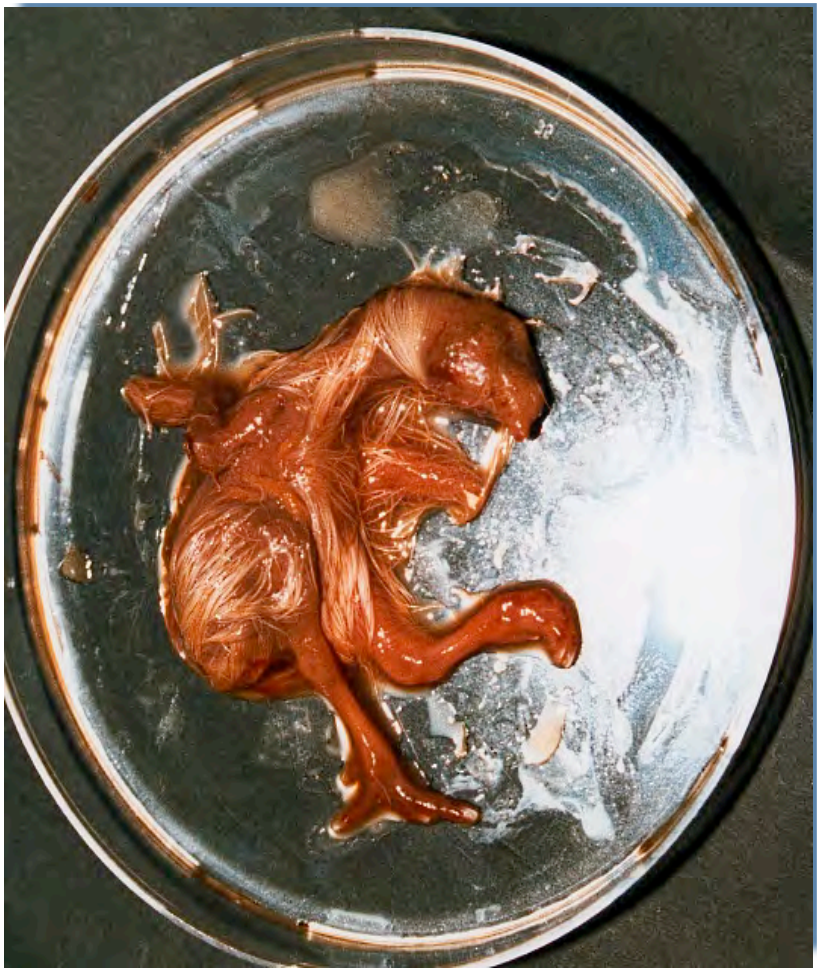


Abnormal beaks and elongated neck

Teratogenic effects of Fusaproliferin



Cephalic dicotomy



Absence of beaks and elongated neck

Ochratoxin A

Nikolic in 2002, on the basis of epidemiological data collected of 1972 and the similarities between the BEN (Balkan Endemic Nephropathy) and the Porcine Nephropathy induced by OTA demonstrated the relation between BEN and Urinary Tract Tumors can be due by the insult of an food contaminant and OTA exposure may be the fungal toxin responsible of the BEN

Ochratoxin A in Foods

The highest amounts of OTA in Food were found in Poland (5,410 ug/kg) and Ceska Republika (3,800 ug/kg). Wheat, maize, barley, beans, potatoes, bread and animal feed also contain OTA

Comparison between crops from ecological farms and conventional farms in Poland show clearly higher OTA contamination in crops from ecological farms, about six times

Similar results were reported in Denmark for the harvest years 1992–1999

Ochratoxin A limits

Some Foods and Beverages, potential vectors, of Ochratoxin A in the Food chain	
Food	**Beverages**
Cereal grains, including corn, wheat, barley, flour, oats, rye, rice, bran and semolina	Wine: red, white and rose
Beans: coffee, cocoa, soy and others	Coffee
Malt	Beer and other malt beverages
Cheese and other dairy products	Milk
Dried fruit	Fruit juices
Pork and pork products	
Poultry and poultry products, including eggs	
Spices	
Nuts	
Peas and other legumes	

The average weekly intake of OTA varies from 130 to 6,489 ng, corresponding to 1.86–92.7 ng/kg body weight (bw) average adult weight of 70 kg or a daily intake of 0.27–13.2 ng/kg bw

The Provisional Tolerable Weekly Intake (PTWI) established by the Joint FAO/WHO Expert Committee on Food Additives and Contaminants is 100 ng/kg bw/week

This assessment has used nephrotoxic effects in pigs to establish acceptable intake levels for OTA

OTA and experiment on mice

Ochratoxin Treatment

OTA was dissolved in 0.1 M Sodium Bicarbonate solution. Pregnant female were injected intraperitoneally (i.p.) with a single dose of OTA at 3.0 mg/Kg body weight, in 100 µl of 0.1 M Bicarbonate, on day 7.5 of gestation and embryo were taken at day 16 (E16) post coitum

Control mice was injected at same time with only 100 µl of sodium bicarbonate solution

OTA and experiment on mice

Embryo analysis

Pregnant, OTA treated, females and control animals were killed by cervical dislocation on gestation day 16

Embryo E16 were removed from uterus

The fixed embryo were investigated to study the morphology and the differentiation defects induced by OTA treatments

After no more than three days the embryo were washed in 70% ethanol, embedded in paraffin and sections of 5 µm were used for *in Situ Hybridization* (ISH)

OTA and experiment on mice

Dlx5 and *Msx1* gene fragments purification

A 216 bp *Dlx5* and 350 bp *Msx1* gene fragments were prepared by reverse transcription polynucleotide chain reaction (RT-PCR) starting from total mRNA extracted from E15 mouse embryo

Total mRNA was extracted from E15 embryo brain and its integrity was verified by electrophoresis. For reverse transcription 2 µg of total RNA in a final volume of 20 µl was reverse-transcribed by Avian Myeloblastosis Virus (AMV) reverse transcriptase in presence of random examer primers at 37 °C per 60 min

PCR amplification of *Dlx5* genes fragment (216 bp), *Msx1* (350 bp) and *Actin* (500 bp) was performed

Differentiation defects induced by OTA treatment

The OTA treatment at a very early gestation time consists in a very dramatic differentiation defect, specially focused in maxillary craniofacial segment

The comparison of the normal head development, in untreated mice with the pups derived from OTA treated, in the same progeny, different malformation degree not apparently correlated to an experimental difference in the procedure or in quantity and timing of drug administration

Results observed

May be possible to hypothesize a generalized high toxicity with a different sensibility of different embryos ranging from little to monstrous malformation

In the same progeny, were observed embryos without severe defect, consisting in loss of symmetry in maxillo-facial formation or a more severe deformity with a loss of an ocular formation with replacement of a large central eye

Results observed

On the other hand, has been identified a progeny with a very high malformation degree probably correlated to a major sensitivity to the drug

Other Embryos of experiment shows a very dramatic malformation on macro encephalocele in frontal region or monstrous loss in head formation as a severe exencephaly with absence of cranial formation

Msx1 *in situ* hybridization

The results of *Dlx5 in Situ Hybridization* hypothesize a negative regulation of genes involved in the craniofacial differentiation after treatment with OTA

On this base also the *Msx1* gene, that acts as transcriptional inhibitor contrary to *Dlx5*, could be hyper expressed or remain constant in a system were is conceivable the inhibition of a genetic pattern

Msx1 *in situ* hybridization

Msx1 in Situ Hybridization **results have not demonstrated differences in the expression rate of this gene**

Msx1 **is poorly expressed in embryos mouse at stage E14 and E16 and the expression of this gene does not increase in mice embryos treated with OTA**

OTA does not interact with *Msx1* promoter and there isn't inhibition induced by a major presence of *Msx1* gene product

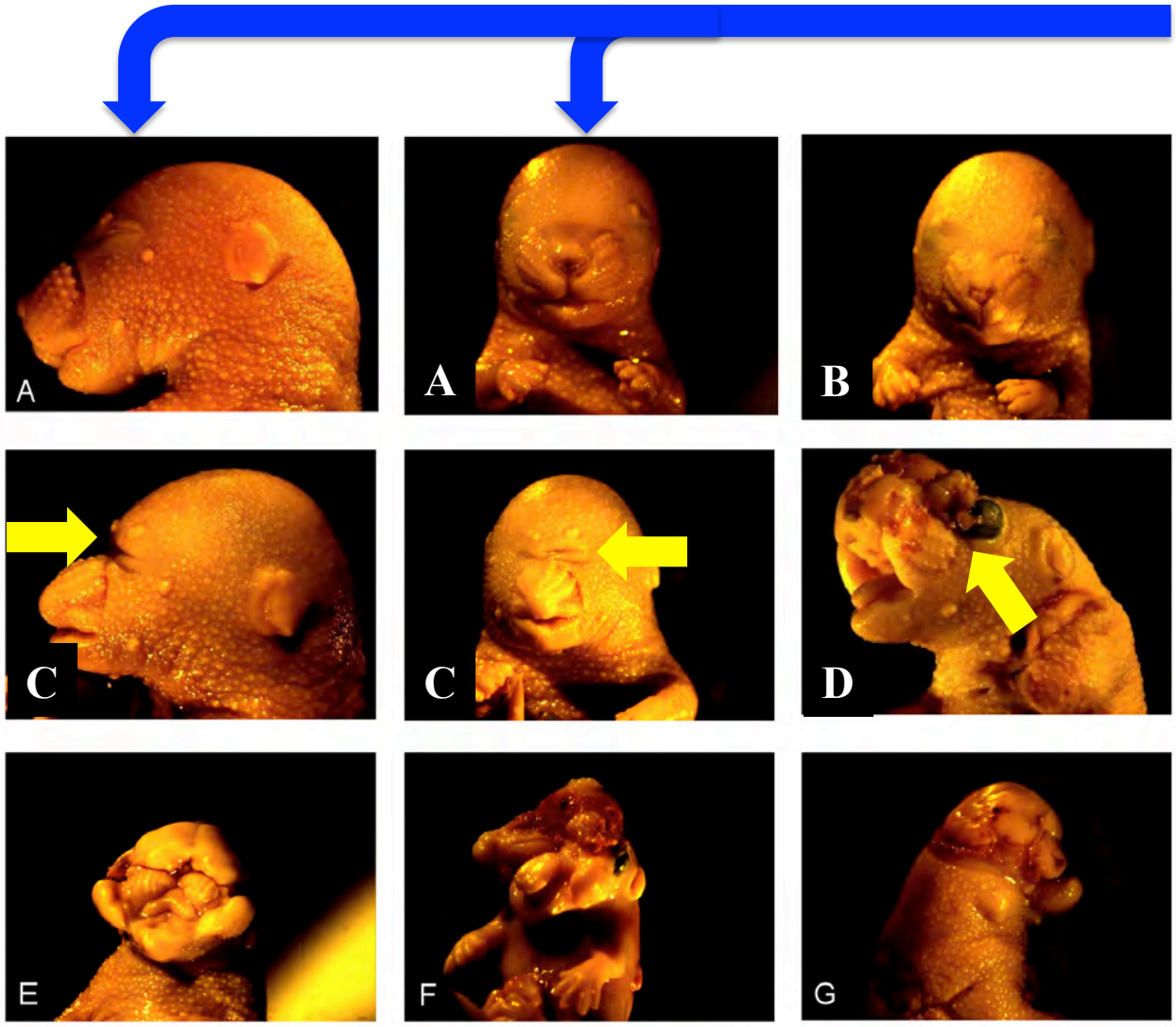
In addition, the genes controlled by *Msx1* are not down regulated by *Msx1* protein after OTA treatment

The teratogenic effects observed, after OTA administration, are probably correlated to the loss of transcriptional activation of genes controlled from the *Dlx5* gene product

Conclusion

A single intra peritoneal dose of OTA (2.75 mg OTA/kg body weight), was teratogenic when given at the 7.5 gestation day, during the major organogenesis period, and if there is a subset of gene, target of OTA, that are responsible for the correct development of a particular body segment, in the aim to explain what is the possible mechanism for the induction of differentiation defects observed after administration of OTA or others mycotoxins

Evidences



E16 embryo following OTA treatments on gestation day 7.5

A: lateral and frontal vision of E16 normal control embryo without OTA treatments

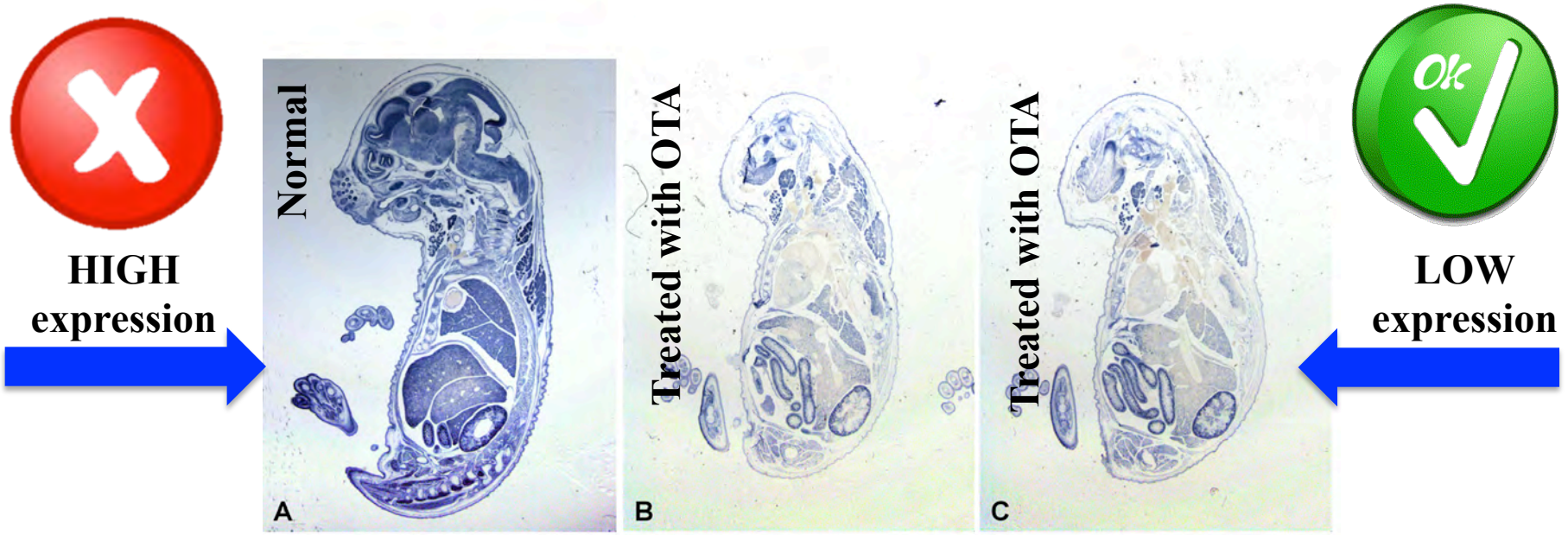
B: OTA treated embryo showing maxillary asymmetry

C: frontal and lateral vision of embryo with synophthalmia and absence of normal eye replaced by a central pseudoocular formation

D: absence of normal head formation with evident encephalocele

E, F, G: loss of head formation with severe exencephaly

Evidences of Dlx5 Expression



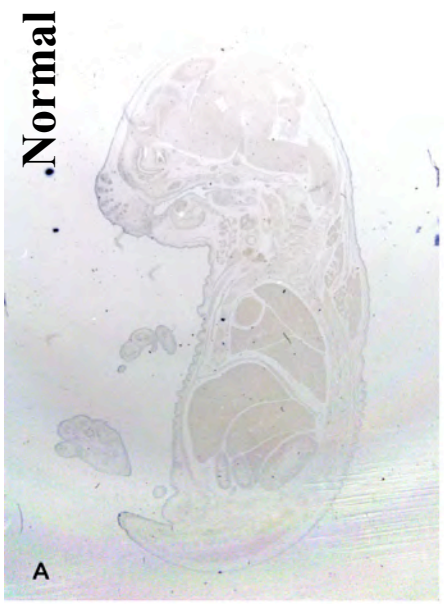
Dlx5 In Situ Hybridization **following OTA treatments on gestation day 7.5**

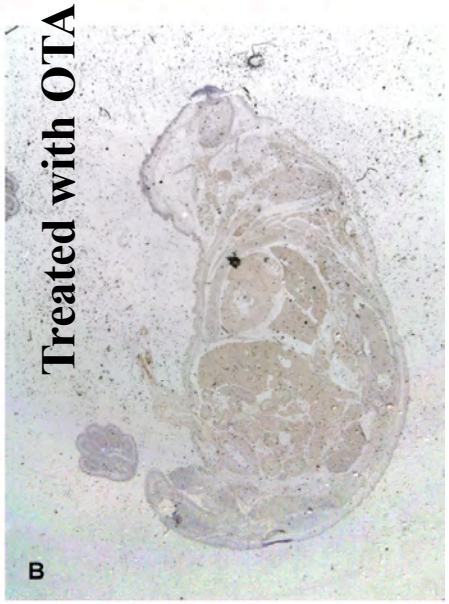
A: E16 normal control embryo without OTA treatments showing high expression of *Dlx5* gene in almost all brain and maxillo-facial structures.

B: *Dlx5 ISH* of OTA treated embryo showing pseudo-ocular formation present a confused or absent *Dlx5* expression overlapping between retina and the hypothalamus inferior part.

C: *Dlx5* ISH of OTA treated embryo with severe craniofacial malformation showing low and confuse *Dlx5* expression, that do not identifies the anatomic structures in cranial formation.

Evidences of Msx1 Expression





***Msx1* is very little expressed in embryo mouse at stage E16, and the expression of this gene in embryo derived from OTA treated mice**

A. untreated control E16 embryo

B: OTA treated E16 embryo

Conclusion

The widely described role of *Dlx5* in the maxillofacial structures formation supports the results, and the shutdown of *Dlx5* gene in embryos, due to OTA, it seems closely related to the observed deformities

Dlx5 gene is target for OTA and its inhibition, directly or indirectly, could origin the deformities related to OTA

It is not demonstrated whether the OTA inhibits the transcription of *Dlx5* binding to the promoter of this gene or acting on other genes, which in turn regulate the expression of *Dlx5*

Conclusion

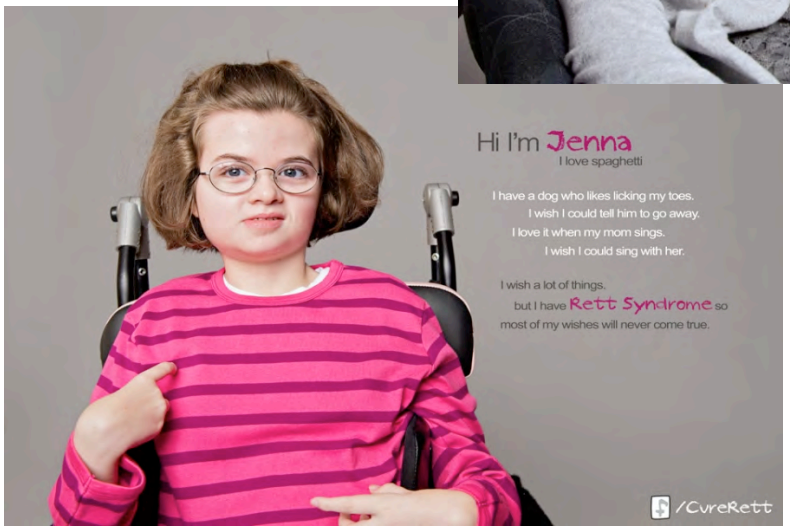
In situ Msx1 **hybridization did not show any change in the expression of this gene between control and the OTA exposed. This does not exclude the involvement of *Msx1* gene in more earlier stage, even considering that at E16 stage, as well as E14 one, this gene is expressed at very low level. Moreover, since the proteins derived from *Msx1* and *Dlx5* can form some eterodimers, the reduced expression of *Dlx5* could reduce the possibility of formation of these eterodimers that are active in regulating the development of maxillo-facial segment**

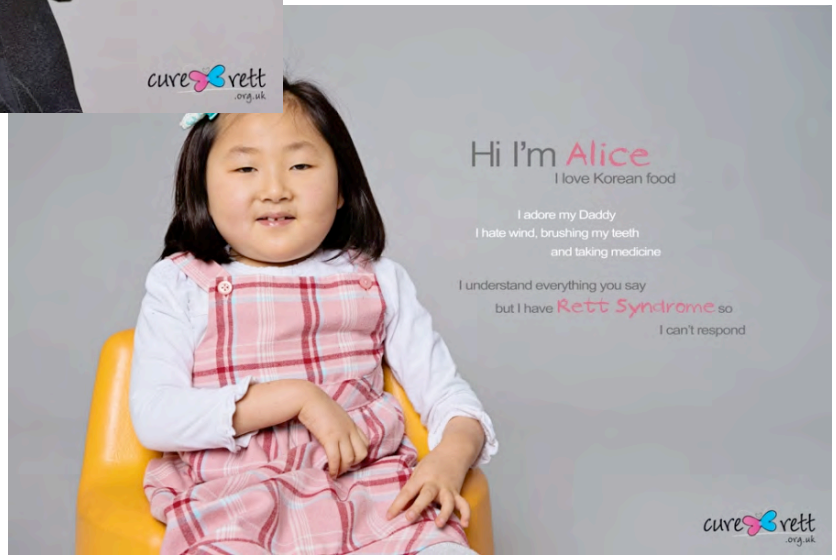
Conclusions

Hypothesis B: the phenotypic changes observed do not depend by *Dlx5* expression inhibition, but by the resulting deregulation of the downstream genes, like those coding for bone morphogenetic proteins, specifically involved in the formation of the palate

A reduced expression of *Dlx5* then could act indirectly by altering the expression of these effector proteins, that results in an alteration of this differentiation pattern

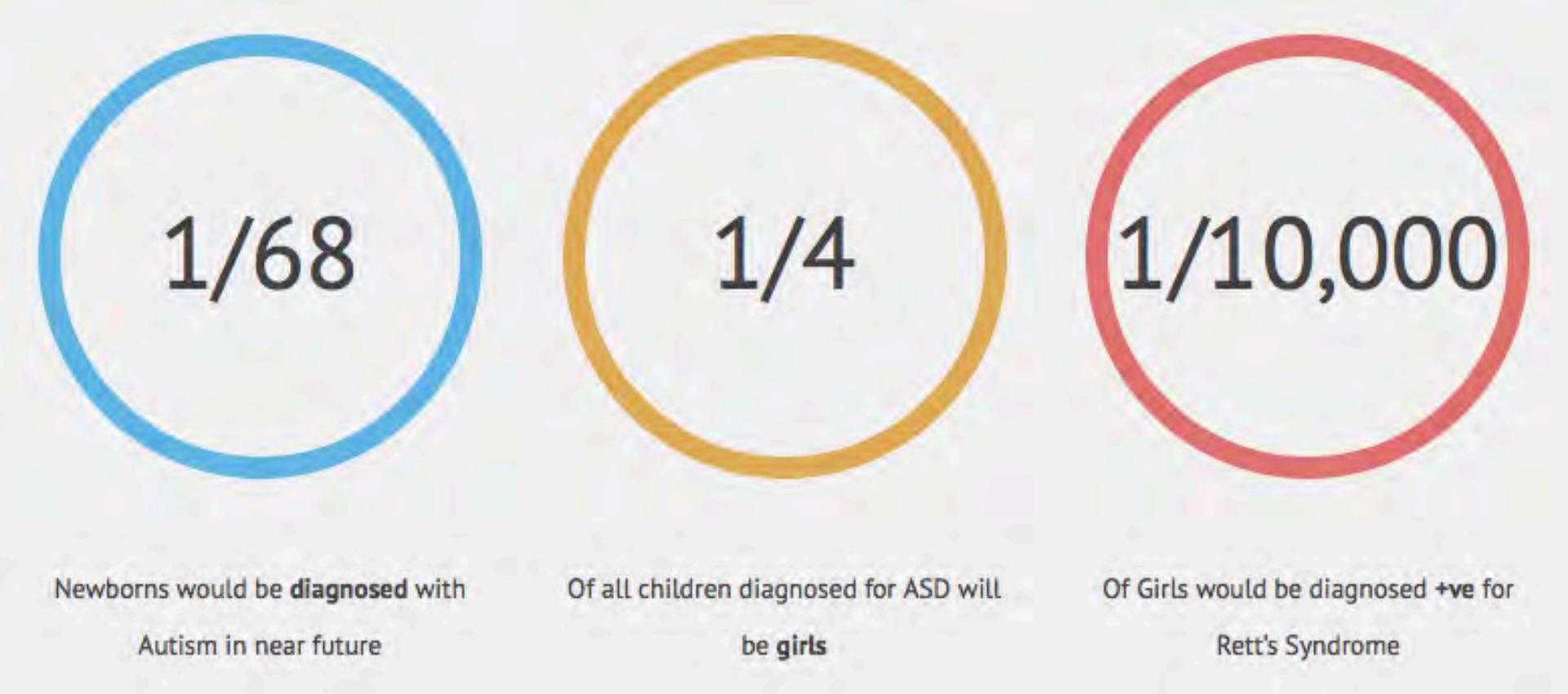
Rett Syndrome





Rett Syndrome

Rett Syndrome incidence in girl population



Rett Syndrome

Rett syndrome is a neurodevelopmental disorder that almost exclusively affects girls.

Children with Rett's syndrome, in the initial stages, tend to show symptoms that are very much similar to early signs of autism.

Much like autism, your child shows normal growth and development followed by a gradual (or sudden) regression of their developmental skills.

Most children diagnosed with Rett Syndrome will have slower head and brain growth which is often an early indication of the disorder. Seizures are quite common as well.

Rett's Syndrome is perhaps the rarest among the major types of Autism and perhaps the only one that could be confirmed through medical tests.

The support level required for girls with Rett's syndrome ranges from Level 2 to Level 3. Jump to the end of the post for the various support levels across the Autism Spectrum Disorder.

Rett Syndrome and DLX5 gene

The gene DLX5 may have a role in Rett Syndrome, a neurological disorder that affects mainly the female sex with an incidence of 1 in 10,000 females and begins in girls 6-18 months of life

The disease is manifested by an arrest of psychomotor development, lack of language, enhanced stereo hands

In 1999 have been identified for the first time mutations of a gene on the X chromosome, MECP2

The expressed protein gene MECP2 can remodel chromatin and this MeCP2 protein binds to the gene DLX5 in the brains of mice

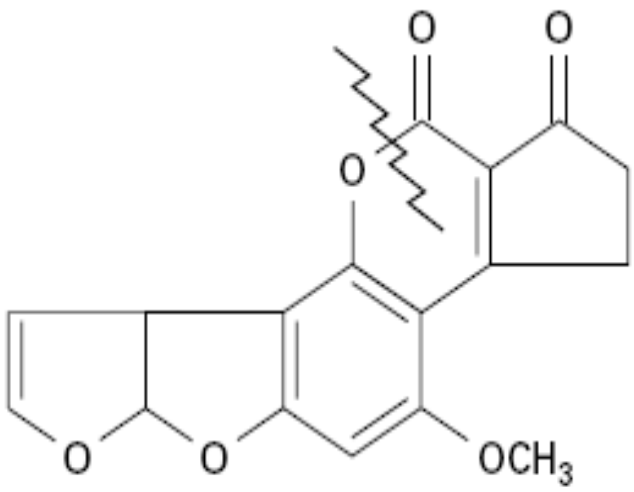
In humans the protein Dlx5 plays an important role in the synthesis of GABA (gamma amino butyric acid), an important neurotransmitter

Subject with Rett Syndrome was observed increased expression of the gene DLX5 However it is not yet fully known how MeCP2 regulates gene DLX5

Aflatoxin

- Aflatoxin B_1 & B_2: produced by *Aspergillus flavus* and *A. parasiticus*
- Aflatoxin G_1 & G_2: produced by *Aspergillus parasiticus*
- FDA: 20-200 ppb
- Aflatoxins are potent toxins. They are well-known for their carcinogenicity
- Aflatoxin B1 is the most important of them, followed by G1 > B2 > G2
- Stability: heat-stable
- Environmental condition: the fungi grow best at approximately 25°C at high relative air humidity (≥80%)
- Aflatoxins are produced at relatively high moisture contents and relatively high temperatures
- Prevention: adequate post-harvest crop-drying

Aflatoxin



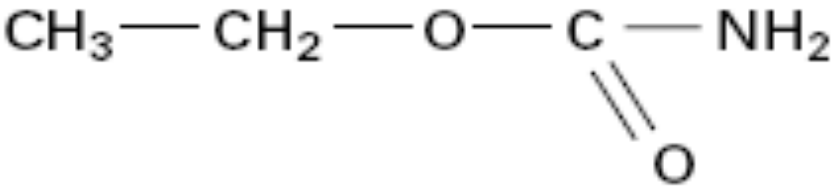
Detoxification of Aflatoxin B1 by opening the lactone ring

Opening of the lactone ring is achieved by treatment with ammonia (NH_4OH) at elevated temperature and pressure, which is applied at industrial scale to reduce in animal feed ingredients AFB1 e.g., groundnut press-cake

At high pH the lactone ring of the aflatoxin molecule is hydrolyzed

Ethyl Carbamate

- **Ethyl carbamate (urethane) is associated with yeast fermented foods and beverages**
- **Metabolism of L-arginine and L-asparagine by yeast**
- **Toxicity and symptoms. Ethyl carbamate is a mutagen as well as a carcinogen**
- ***Environmental condition: Light, Heat, Precursor: ethanol, HCN***
- **FAO/WHO → 10 ppb for softdrinks**



Ethyl carbamate (Urethane)

Occurrence of Ethyl Carbamate

Product	Number of samples	Average level (ppb)	Range (ppb)
Yogurt	12	0.4	ND–4
Cider	8	0.6	ND–4
Bread	30	1.7	ND–8
Malt beverages	69	1.8	ND–13
Bread, toasted	9	5.2	2–14
Soya sauce	12	18	ND–84
Wine	6	18	7–40
Sake	11	52	3–116

Note: ND = not detectable.

[1] Data found in literature.

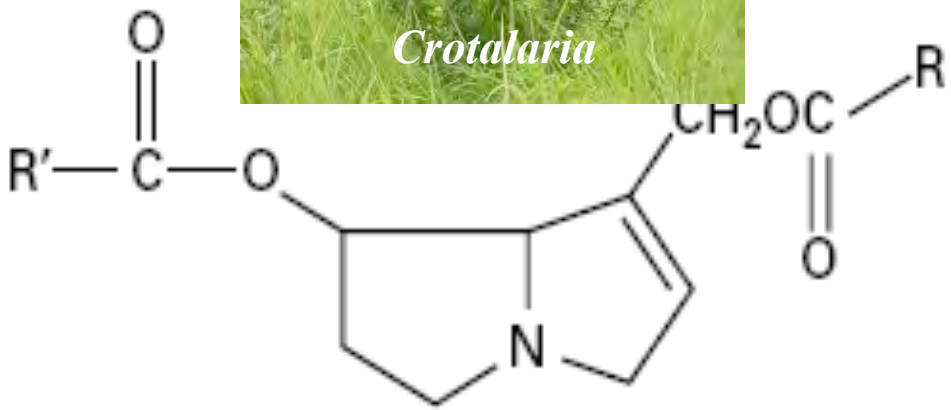
Ethyl Carbamate

Prevention: levels of the precursors by enzymatic treatment, selection of yeast strains, control of fermentation conditions and treatment of EC may be useful in keeping its levels at a minimum

Toxic Substances in Plants

Pyrrolizidine alkaloids

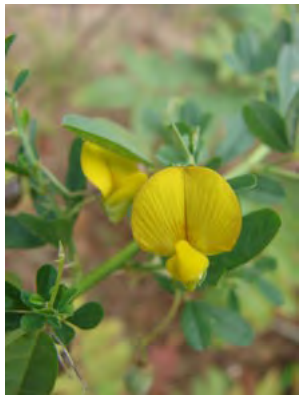
produced by the genera *Senecio, Crotalaria and Heliotropium* the cause of acute liver damage and vein lesions → liver cancer



Pyrrolizidine alkaloids

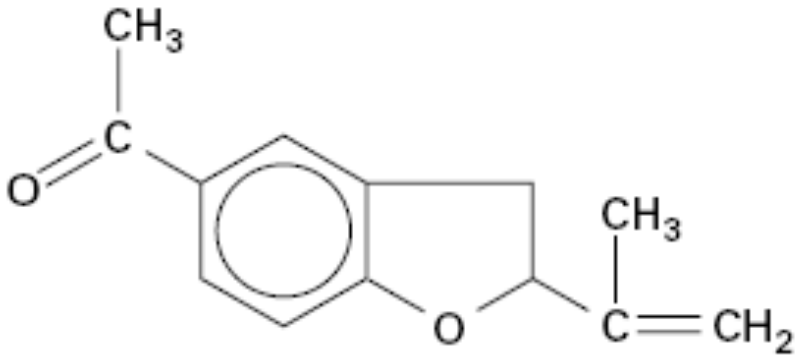
Toxic Substances in Plants

- **In India, millet, the principal cereal in the diet, appeared to be heavily contaminated with *Crotalaria seeds.***
- **The alkaloid content of the seeds was estimated at 5.3 mg/g**
- **In Afghanistan, the consumption of wheat bread heavily contaminated with *Heliotropium* seeds was found to be the cause of the intoxication.**



Toxic Substances of Animal origin

- **The toxin originated from either white snakeroot *(Polygonum)*, *or the rayless* goldenrod *(Solidago)*→ outbreaks of "milk sickness"**
- **the major toxic component appeared to be tremetone**



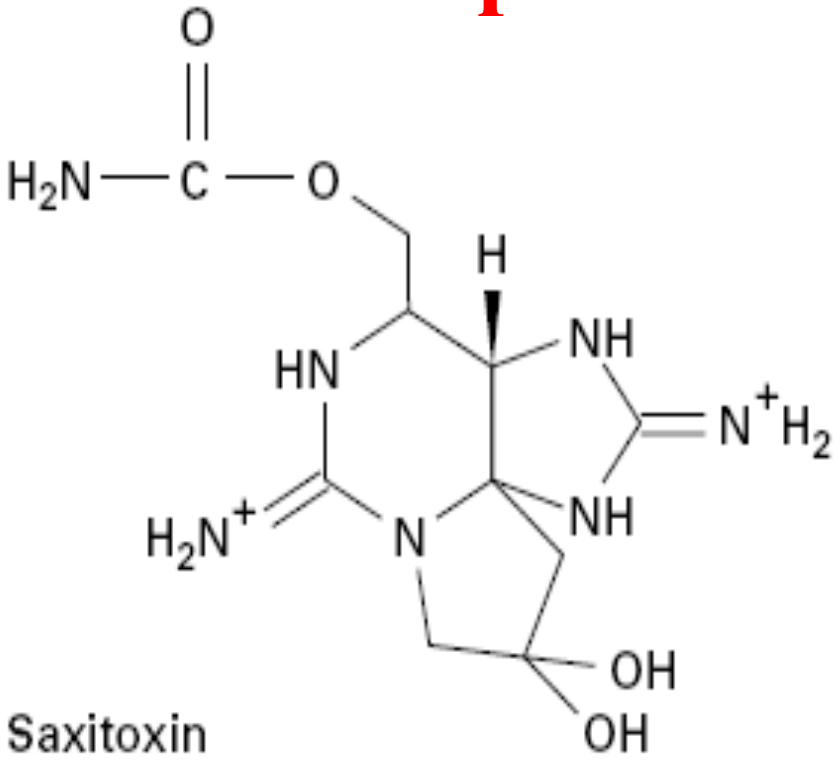
Tremetone

Toxic Substances by Animals

- The symptoms were weakness, followed by anorexia, abdominal pain, vomiting, muscle tremor, and coma, and eventually death
- The mortality rate was between 10 and 25%

Natural toxins in aquatic organisms

- **Shellfish that have become contaminated with a toxin or group of toxins from the ingestion of toxic plankton, in particular toxic dinoflagellates which produced SAXITOXIN**



Saxitoxin

Natural toxins in aquatic organisms

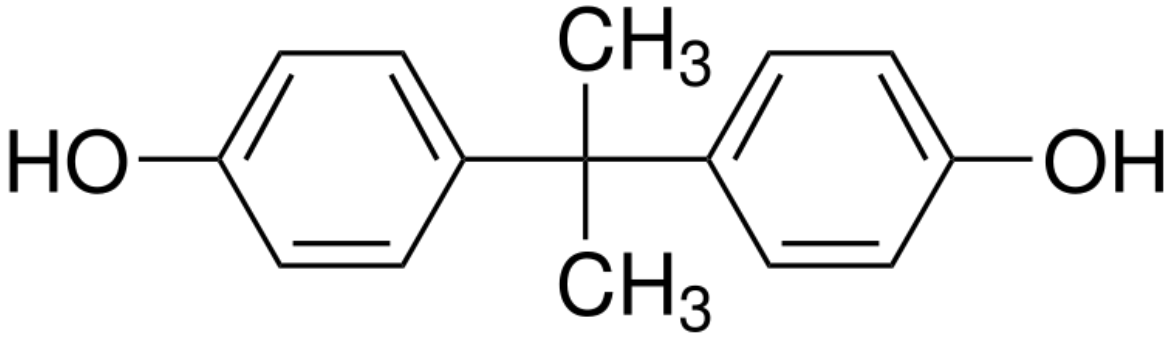
- **Symptoms: burning in face, lips, tongue and headache. These symptoms develop within 30 minutes after ingestion**
- **Death, preceded by respiratory paralysis, occurs within 12 hours**
- **Contamination and poisoning is highest during red tide → the sea sometimes suddenly becomes colored, as a result of dinoflagellate bloom**
- **Also be yellowish, brownish, greenish, and bluish in color**
- **Prevention: cooking (destroys up to 70% of the toxin) and pan-frying destroys(> 70%)**

Bisphenol A


10 Canned Foods to Avoid to Reduce BPA Exposure
1 Coconut milk
2 Soup
3 Meat
4 Vegetables
5 Meals (e.g., ravioli in sauce)
6 Juice
7 Fish
8 Beans
9 Meal-replacement drinks
10 Fruit
*based on testing of more than 300 products
www.breastcancerfund

What is Bisphenol A ?

- **BPA**



- **First synthesized in 1891**
  Condensation of acetone with phenol
- **In 1930s it was found to be an artificial estrogen (like to DES)**

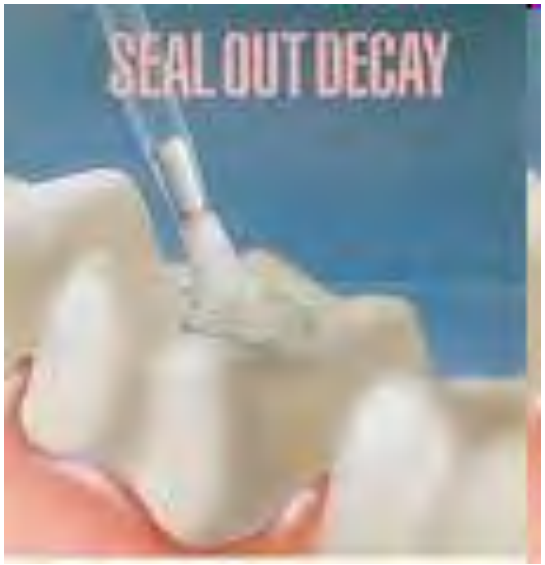
BPA in plastics

- In the 1940s and 1950s it was discovered that BPA can be used in the synthesis of polycarbonate and epoxy resins
- Currently over 2 million tons are produced world wide
- In the U.S. it is made by Bayer, Dow, GE, Hexion, and Sunoco
- Also used as a polymerization inhibitor in PVC

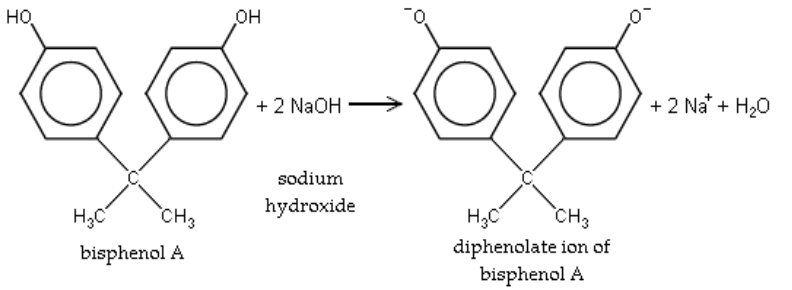
Where do we find BPA ?

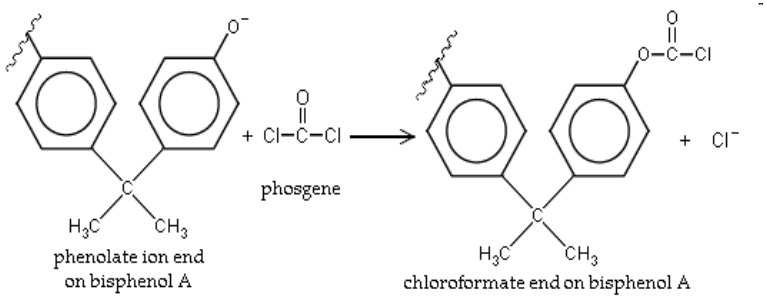
Common uses

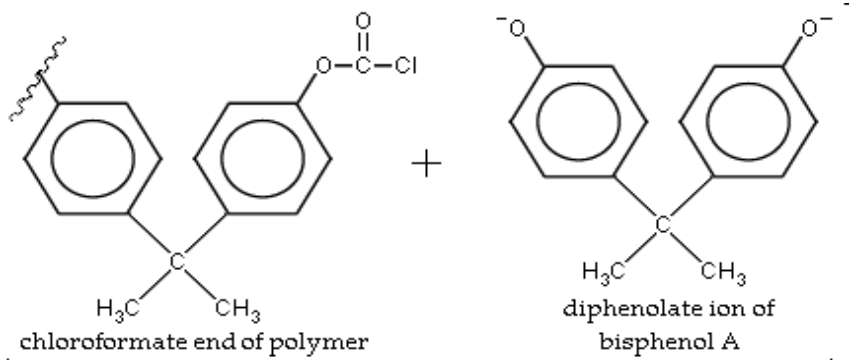
- Dental sealants
- CDs
- Eye glasses
- Food and beverage can coatings
- Reusable water and baby bottles
- Film
- Water pipes and sealants in water towers
- Etc.

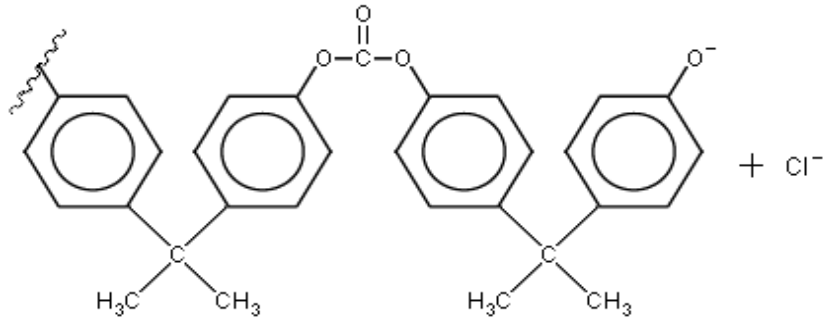


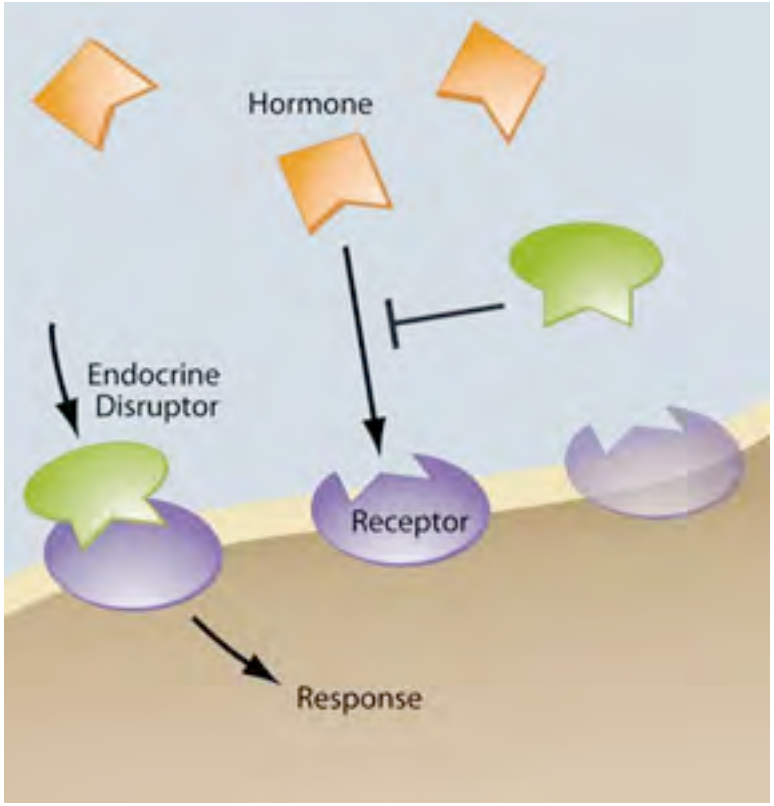
Bisphenol A


HO
OH
+ 2 NaOH
sodium hydroxide
+ 2 Na+ + H2O
bisphenol A
diphenolate ion of bisphenol A

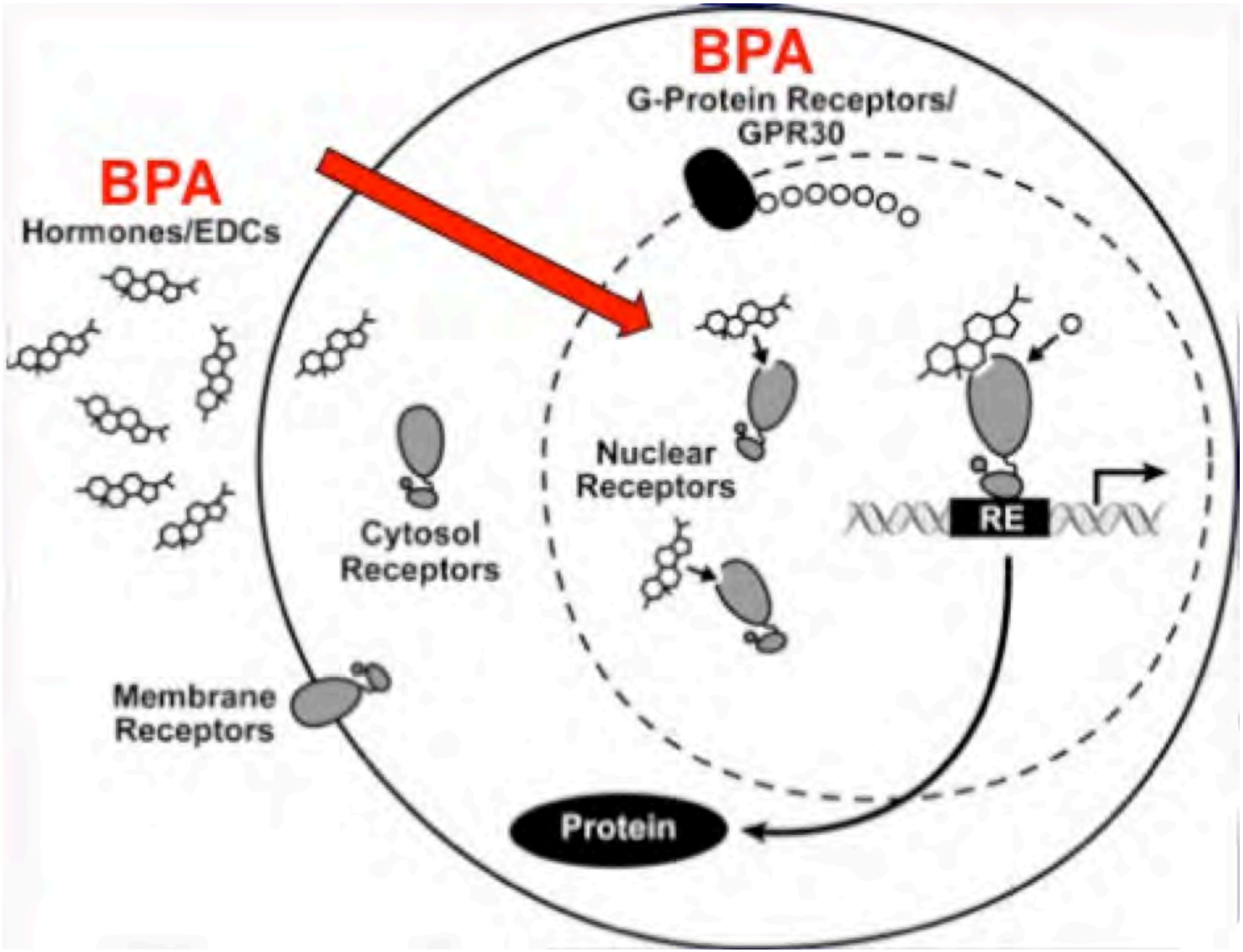

+ Cl–C–Cl
phosgene
+ Cl⁻
phenolate ion end on bisphenol A
chloroformate end on bisphenol A


chloroformate end of polymer
diphenolate ion of bisphenol A


+ Cl⁻
polycarbonate polymer being extended

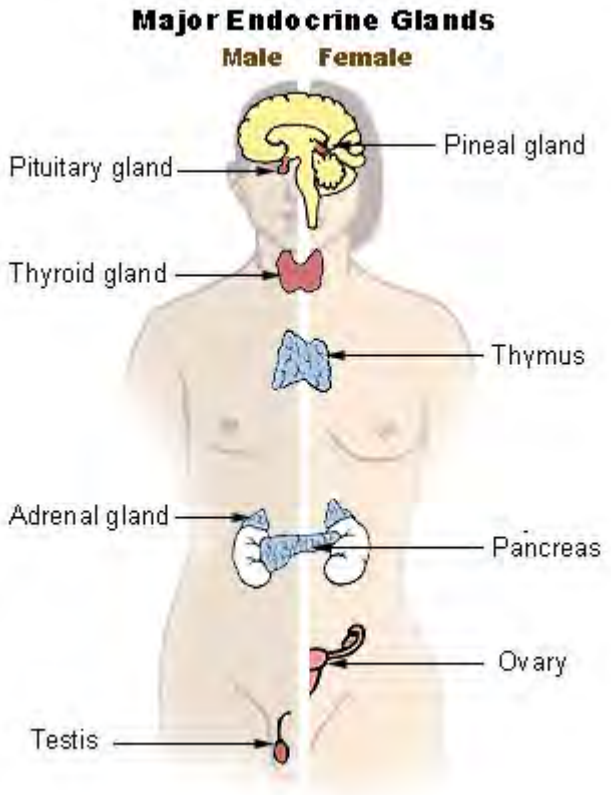

Hormone
Endocrine Disruptor
Receptor
Response

BPA Endocrine Disruptor



Possible Health concerns

- **BPA is an endocrine disruptor**
- **An endocrine disruptor is a chemical that mimics hormones**
- **Potentially causes problems with the reproductive system**
- ***Our Stolen Future***



Effects of Bisphenol A

Dose (µg/kg/day)	Effects (measured in studies of mice or rats, descriptions are from Environmental Working Group)	Study Year
0.025	Permanent changes to genital tract	2005
0.025	Changes in breast tissue that predispose cells to hormones and carcinogens	2005
2	increased prostate weight 30%	1997
2	lower bodyweight, increase of anogenital distance in both genders, signs of early puberty and longer estrus.	2002
2.4	Decline in testicular testosterone	2004
2.5	Breast cells predisposed to cancer	2007
10	Prostate cells more sensitive to hormones and cancer	2006
10	Decreased maternal behaviors	2002
30	Reversed the normal sex differences in brain structure and behavior	2003
50	U.S. human exposure limit (not a result from an animal study, but a guideline set by EPA)	1998

FDA position

- The FDA evaluation only uses tests where the BPA is ingested
- Assumes that BPA is much more easily metabolized by primates than mice
- Determined the No Observable Adverse Effect Level to be 5 mg/kg/day
- The tests used tend to favor tests performed by industry

Other Agencies

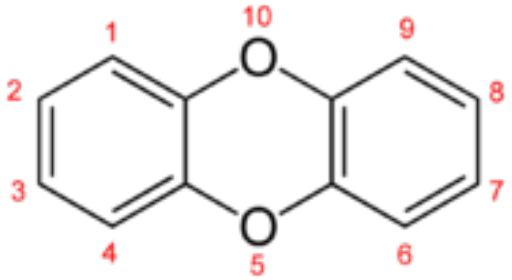
- The European Food Safety Authority established the Tolerable Daily Intake to be 50 µg/kg/day
- Japan has said there is no risk
- Canada now states that BPA does not pose a risk

Dioxins

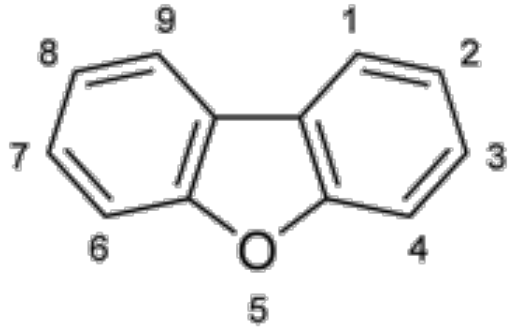
***Dioxins*: is a family of compounds including PCDDs & PCDFs with Cloro atoms at any of the 8 sites on the benzene rings**

→ 210 possible congeners

PCDD: Polychlorinated dibenzo-*p*-dioxin (75 congeners)

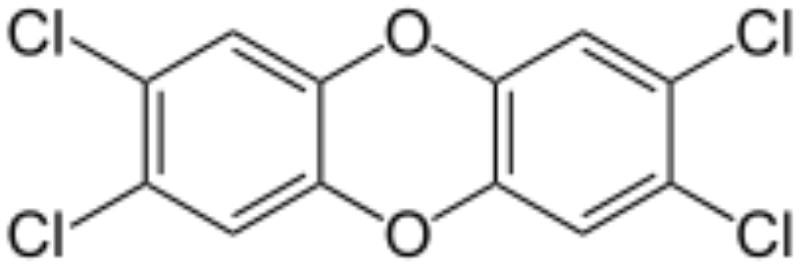


PCDF: Polychlorinated dibenzofurans (135 congeners)



Dioxins

The most important Dioxin is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD):



This compound is the most toxic chemical compound ever evaluated by the EPA

TCDD half-life in the body is 7 years

In common with other POPs, dioxins have:

- **low vapour pressures**
- **low solubility in water (hydrophobic)**
- **good solubility in organic solvents, oils, and fats (lipophilic)**
- **bioconcentration**

Source of Dioxins

Dioxins are produced when organic material is burned in presence of chlorine (from Cl^- ion or an organochlorine compound)

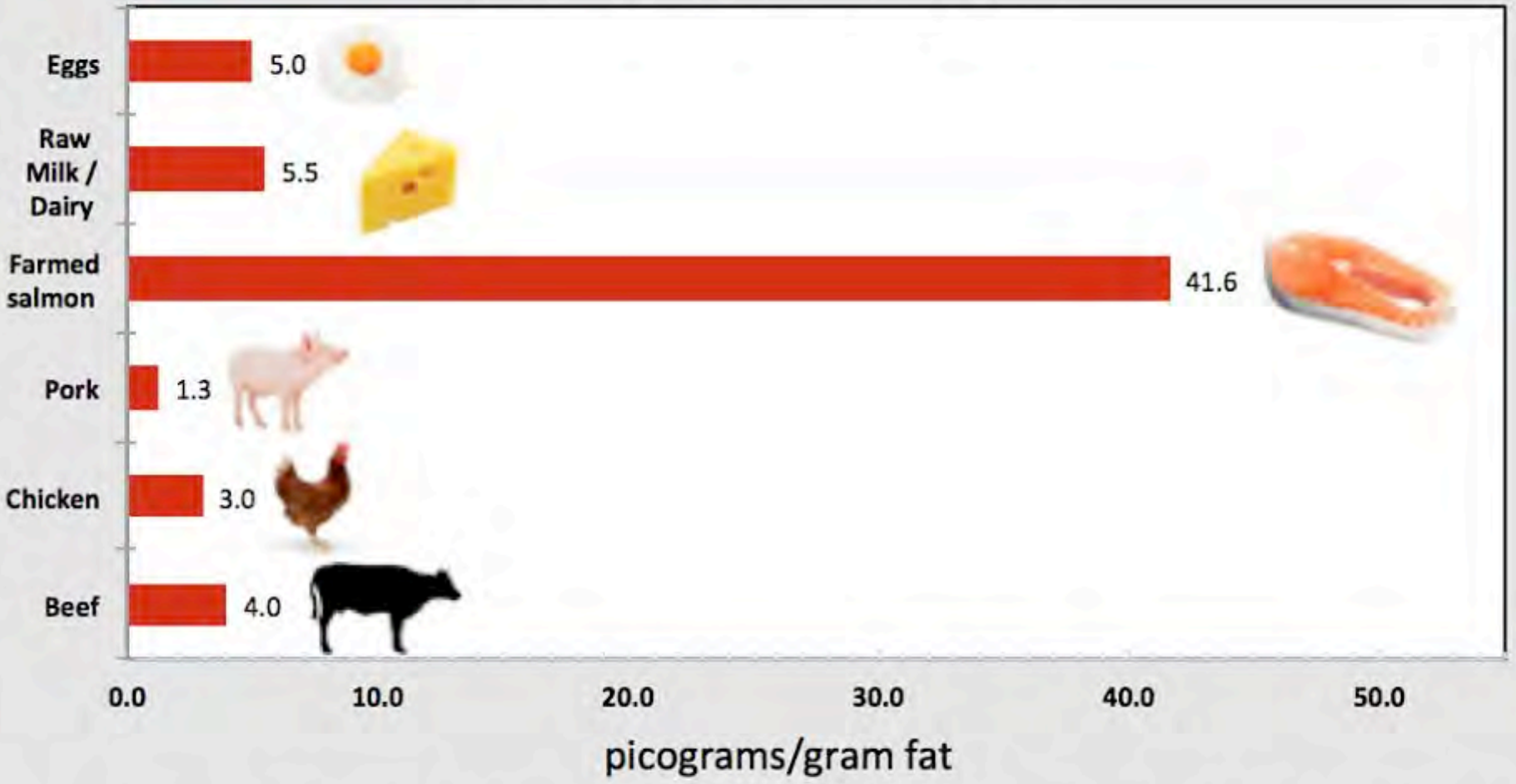
Widely produced and the major sources are:

- **incineration of municipal & medical wastes**
- **coal-fired utilities**
- **metal smelting**
- **diesel trucks**
- **burning treated wood**
- **misapplication of sewage sludge**
- **bleaching of paper fibres and textiles**

Dioxins are largely anthropogenic

Most dioxins (>99%) are found in the topsoil

Source of Dioxins



Source data: EU Commission Regulation (EU) No 1259/2011 of 2/12/2011, Maximum Allowable levels for sale in the EU Market
Farmed Salmon contains 15.6% average total fat in the filet, from the Norwegian National Institute of Nutrition and Seafood Research Seafood Database. Accessed 25 October 2013

Dioxins and the environment

Dioxins are widespread – all people are exposed to a low background concentrations of dioxins

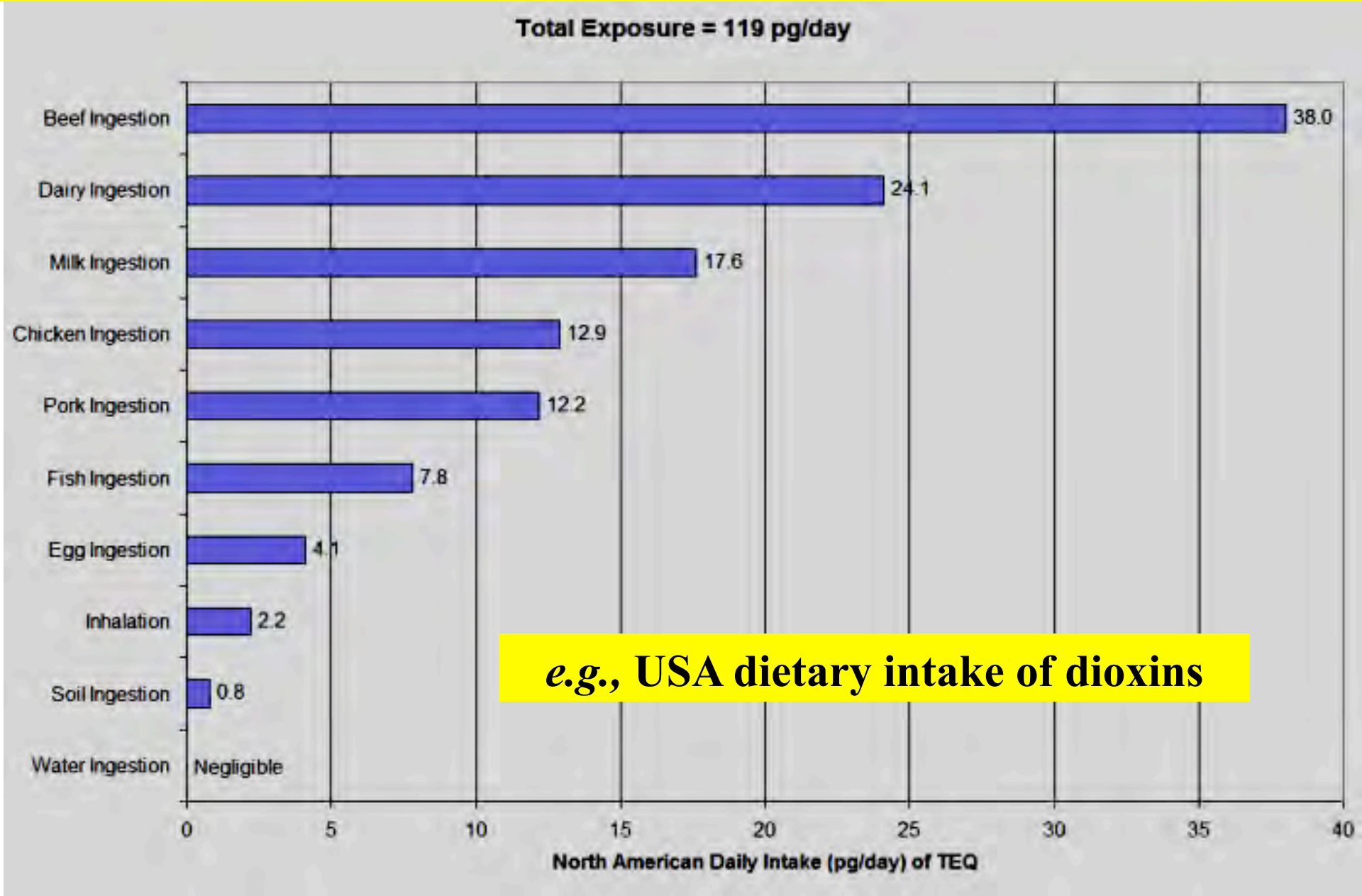
Human exposure:

- **> 90% of human intake is through food**
- **fish, meat, and dairy are most important sources**
- **concern about dioxin exposure in infants and breast-fed children owing to greatly elevated intake of dioxins**

Besides *background exposure*, people may also be exposed to dioxins through *accidental exposure* (e.g., the Seveso Disaster) or *occupational exposure* (e.g., in some chemical industries).

Mean intake: 50 – 200 pg/day for an adult (60 kg)

Dioxins and the environment



***e.g.*, USA dietary intake of dioxins**

Dioxins and the environment

Current sources account for only 10-30% of the total dioxins

other sources ?

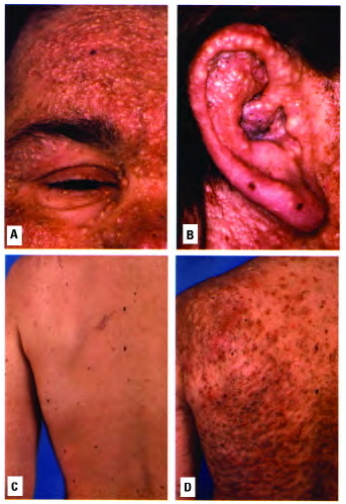
volatilisation and recondensing of dioxins ?

Deposition of emissions on farmland and subsequent uptake into the food supply is a particular cause of concern

Health effects of Dioxins

Adverse health effects associated with dioxins include:

- **cancer ???**
- **immune system damage**
- **birth defects**
- **diabetes**
- **endocrine disruption**
- **chloracne**



Different congeners have different toxicities.

Relate toxicities using the *Toxic Equivalence Factor* (*TEQ*)

This factor is defined as 1 for TCDD

Tolerable Daily Intake (TDI) =

10 pg / kg body weight (TCDD)

Dioxin poisoning

The Seveso Disaster (July 1976)

An industrial accident in the Italian town of Seveso, 25 km from Milan, resulted in the highest exposure of residential population to TCDD

About 800 residents were exposed to high TCDD concentrations

Over 3,000 animals died within days of the accident

Emergency slaughtering of tens of thousands of animals was undertaken to prevent the introduction of dioxins into the food chain

Complete evacuation of nearby area

- **hundreds of cases of chloracne & skin lesions**

Subsequent costs include compensation to victims & clean up costs

Dioxin poisoning

Victor Yushchenko (September 2004)

Ukrainian opposition leader, Victor Yushchenko, became acutely ill during the 2004 Ukrainian elections

Hospitalised in Vienna with "*acute pancreatitus*"

Dutch toxicologist suggested testing for Dioxin levels

Dioxin concentrations found to be 1,000 times higher than normal concentration

Hvala vam na pozivu za upoznati ovaj prekrasan grad Zagreb i hvala vam na pažnji i dobrodošli