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Palytoxin and Okadaic acid as seafood contaminants: risk characterization

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INTRODUCTION

Marine toxins are non-proteinaceous and heat stable compounds produced by different species of unicellular algae, ranging from dinoflagellates, which produce the majority of these compounds, to diatoms and even cyanobacteria. These toxins can be accumulated in edible marine organisms, such as fish, shellfish, crustaceans and echinoderms and the consumption of contaminated seafood can cause different types of poisonings in humans, which clinical signs may range from gastrointestinal up to neurological ones [1, 2, 3, 4, 5]. Gill et al. [5] calculated that 60,000 poisoning events are recorded every year, 1.5% of which associated with mortality.

When poisoning occurs, its severity, depends both on the type of toxin contaminating seafood, the quantity of eaten seafood and on its toxin content. At present there is no feasible way of preventing shellfish from accumulating toxins or of removing such toxins once the shellfish have been harvested. This means that these compounds must undergo a strict risk assessment in order to define regulatory limits for safeguarding human health. Appropriate rules of hygiene should be in place to impede the serious dangers for humans that may be associated with this type of food. Increasing interest is being shown in marine algal toxins because of their commercial impact, their influence on the safety of seafood and potential medical and other applications. One drawback for research using toxins is the problem of obtaining sufficient quantities, given their limited availability.

Seventy out of the ninety species of toxin-producing marine microalgae are dinoflagellates. Among the 5000 existing marine algal species, approximately 300 can sometimes occur during microalgae proliferations, known as algal blooms. Cysts can remain viable for long periods of time until favourable environmental conditions allow

them to germinate. While no definite causes have been identified, it is recently speculated that environmental factors such as climate changes, with rising ocean temperatures, reduction in pH and growing availability of nitrate, increasingly encourage the formation of algal proliferation. Moreover, toxic species of algae may be spreading into new areas with the burgeoning of marine traffic and selling of spats for cultivation in other countries. Another factor is that nowadays the consumption of seafood is much more widespread than in the past, while more stringent regulatory standards and monitoring to recognize toxins and diagnose human illnesses may also contribute to this apparently increasing trend [6, 7, 8].

Climate changes have made it nearly impossible to predict the blooms of harmful algae and the consequent seafood contamination. In general, algal blooms occur when heated sweet water creates a differential layer from the colder seawater into which it runs into. The lower, colder layer becomes what is known as the pycnocline, once the fast growing algae deprive the upper layer of nutrients leaving phospohorus and nitrogen alone below the interface [9]. In these conditions motile dinoflagellates thrive; taking up nutrients from the lower levels and then migrating again to the upper, warmer level causing blooms, which suddenly appear on the surface waters that are devoid of nutrients and cannot support this exaggerated growth. An additional factor that could increase algal blooms formation seems to be domestic, industrial and agricultural residues dumped into seawater. Trace materials, acidic precipitation can also set a prolific substrate for additional algal proliferation. Thus, increased incidence seems to be linked to environmental conditions- leading to higher temperatures and higher algal proliferation rates-, increase in monitoring as well as increase in aquaculture activity, and subsequent health monitoring for human consumption, has increase awareness, but also monitoring of

these phenomenon [10]. It is still not known what triggers the toxin production that leads to outbreaks and it is essential to conduct further research seeking advice from scientists with expertise.

Historically, marine algal toxins classification was based on the clinical symptoms they cause. At present, they are classified on the basis of their chemical structure into the flowing groups: saxitoxin (STX) group, brevetoxins group, ciguatoxins, domoic acid (DA) group, azaspiracid (AZA) group, okadaic acid (OA) group, pectenotoxin (PTX) group, yessotoxin (YTX) group, palytoxins (PLTXs) and cyclic imines group.

Saxitoxin (STX) group

Saxitoxin and its analogues are a family of more than 30 hydrophilic toxins which provoke a syndrome known as paralytic shellfish poisoning. Saxitoxin and its derivatives, including gonyautoxins, are tetrahydropurines, which chemical structure is represented by two guanidinium moieties joined in a stable azaketal linkage. They can be divided in four subgroups on the basis of particular substituents in position C6: the carbamate toxins (i.e. saxotoxin, STX; neosaxitoxin and gonyuatoxins), the N-sulfo-carbomoyl toxins (i.e. gonyautoxin5 and -6, GNTX5 and-6; C1-4), the decarbomoyl toxins (dc-STX, dc-neoSTX, dcGNTX1-4) and the deoxydecarbomoyl toxins (doSTX, doGNTX1). The toxins are produced by dinoflagellates of the genus *Alexandrium*, *Pyrodinium bahamense* var. *compressum* and *Gymnodinium catenatum*. These toxins are accumulated in different species of shellfish by filter feeding the toxin producing algae. The consumption of contaminated shellfish cause a potentially lethal poisoning, which clinical signs vary from tingling, perioral paresthesias, numbness, weakness to respiratory arrest which can cause fatalities within 2 to 12 hours from ingestion. The mechanism of action of these toxins is

related to the blockage of the voltage gated sodium channel, innibiting the entrance of sodium ions into the cells and blocking the propagation of the action potential [1, 9, 10, 11].

Brevetoxins group

Brevetoxins are lipid-soluble polyethers causing neurological shellfish poisoning (NSP). They are polycyclic polyethers grouped in two types (A and B) on the basis of their backbone of 10 (type A) or 11 (type B) transfused rings; there is also a form in which the lactone (A ring) is open. These toxins are produced by dinoflagellates of the genus *Karenia*, mainly *K. breve* (previously named *Gymnodinium breve*), which was involved in the "red tide" blooms along the Florida coast and the Gulf of Mexico. These blooms caused massive mortality of different marine organisms and also epidoses of respiratory distress in humans after inhalation of aerosolized seawater as well as eye and skin irritations after swimming in the sea. Shellfish are resistant to brevetoxins and can accumulate these compounds. In addition, shellfish can metabolize brevetoxins, producing a variety of metabolites many of which are more polar than the toxins produced by microalgae. Contaminated shellfish ingestion can provoke the illness named ASP, characterized by acute gastrointestinal and neurological symptoms (nausea, vomiting, diarrhoea, chills, sweats, headache, muscle weakness and joint pain, paraesthesia, arrhythmias, tachipnea, mydriasis, hypothermia, double vision, and troubles in talking and swallowing), which recover two-three days. Brevetoxins effects are caused by selective binding on the cell membrane at the voltage gated sodium channel at site 5, which leads to channel activation at normal resting potential. This action influences the memebrane properties of excitable cells, inducing a neurotoxic effect [1, 9, 10, 12].

Ciguatoxins

Ciguatoxins are lipid-soluble polyethers having a backbone of 10-14 rings transfused by ether linkages into a ladder-like structure that resembles brevetoxins structure. They cause ciguatera fish poisoning (CSP), a tropical illness caused by contaminated fish consumption, characterized by moderate-severe gastrointestinal symptoms (vomiting, diarrhoea, abdominal cramps), neurological symptoms (myalgia, paraesthesia, cold allodynia and ataxia), pruritus and sometimes cardiovascular effects. The proportion of gastrointestinal and neurological symptoms depends on the region: gastrointestinal symptoms, which develop within 2-12 hours from the contaminated fish ingestion, characterize the first stage of this poisoning and are predominant in Caribbean cases of ciguatera, while neurological symptoms are predominant in the Pacific area and develop over 24 hours. Anyway, the onset can vary even in patients who eat the same fish. Cold allodynia, a dysesthesia that provokes a burning sensation on contact with cold objects, is one of the most typical symptoms of ciguatera. Ciguatoxins, produced by the benthic epiphytic dinoflagellate *Gambierdiscus toxicus*, accumulate through the food chain, from small herbivorous fish grazing on coral reefs and through the food chain, by their ingestion by carnivorous species. Ciguatoxins produced by algae can also be biotransformed in the fish. Effects are a direct result of their effect on all excitable membranes which generate and propagate action potentials by affinity binding to voltage sensitive sodium channels, causing their opening at normal resting membrane potential and leading to spontaneous action potentials in excitable cells. They also induce mobilization of intracellular calcium ions and cell swelling. This leads to cell and mitochondrial swelling and bleb formation on cell membranes. Variations in nerve

conduction and positive ionotropic effect as well as disruption of ion exchange systems in the gastrointestinal epithelium were observed [1, 9, 10, 13].

Domoic acid (DA) group

Domoid acid (DA) and its isomers (isodomoic acids A–H and DA 5'-diastereoisomer) are hydrophilic tricarboxylic aminoacids responsible for the syndrome known as amnesic shellfish poisoning (ASP). Domoic acid is produced by *Pseudo-nitzschia* marine diatoms, such as *P. multiseries*, *P. pseudodelicatissima* and *P. australis*, while *P. navis-varingica* has been found to produce isodomoic acids A and B as main toxins. These toxins acid can be accumulated in blue mussels and other shellfish, such as cockles, razor clams, scallops, but has beed detected also in crabs, furrow shell and anchovies. The ingestion of seafood contaminated by these toxins cause a series of clinical signs and symptoms, which include gastrointestinal symptoms within 24 h (abdominal cramps, vomiting and diarrhoea) and/or neurological symptoms within 48 h (headache, disorientation, amnesia, lethargy and sometimes convulsions and coma). Mortality was also recorded in some cases. The toxic effect of DA is consequent to its action on excitatory aminoacid receptors and synaptic transmission. In particular, its effect is mediated by pre- and postsynaptic receptors in a manner similar to the excitatory aminoacid kainic acid, by binding to brain kainate receptors and opening channels to calcium ions and inducing cellular lethality [1, 9, 10, 14].

Azaspiracid (AZA) group

Azaspiracids are a group of lipophilic polyethers characterized by a unique spiral ring assembly, a cyclic amine and a carboxylic residue. They are produced by the dinoflagellate *Azodinium spinosum* and were found in different species of shellfish, also as metabolites formed by these marine organisms. Azaspiracids are the causative agents of the human illness named azaspiracid poisoning (AZP), which symptoms are similar to those observed in DSP poisoning, and include nausea, vimiting, severe diarrhoea and gastric gramps. Reports on AZP and/or contaminated shellfish occurred in some European countries (including Ireland, theUnited Kingdom, Norway, the Netherlands, France, Spain and Italy) but the toxins have been detected also in northwestern Africa, eastern Canada, and South America. The mechanism at the basis of AZP is still not defined, even though *in vitro* studies evidenced several effects, such as cytoskeletal alterations and decreased F-actin pools and tight junction protein changes, with increased paracellular permeability of intestinal epithelial cells [1, 9, 10, 15].

Okadaic acid (OA) group

Okadaic acid and its analogues dinophysistoxins are lipophilic polyethers cusing a syndrome known as diarrhoeic shellfish poisoning (DSP) after mussels, clams, scallops and oysters consumption. These toxins are produced by dinoflagellates of the genus *Dinophysis*, but have also been identified in cultured dinoflagellates of the genus *Prorocentrum* but these species were sporadically detected in phytoplankton during DSP outbreacks. DSP cases were reported in different areas, but the most affected areas are Europe and Japan, and to a lesser extent North and South America, Thailand, Australia and New Zealand. Toxin production may vary considerably among dinoflagellates and among regional and seasonal morphotypes in the same species [16]. Clinical signs include

manly diarrhoea, nausea, vomiting and abdominal pain starting as early as 30 minutes after ingestion of contaminated shellfish to approximately 72 hours. OA and its analogue dinophysistoxin-1 are known to inhibit protein phosphates, mainly protein phosphatase 1 and 2A, enzymes involved in the dephosphorylation of serine/threonine residues of eukaryotic cell proteins. As a consequence, an increased level in phosphorylated proteins occurs in cells. The diarrhea and the degenerative changes in absorptive epithelium of small intestine induced by these toxins have been attributed to an increased level of the phosphorylated proteins controlling ions secretion in intestinal cells and of cytoskeletal and/or junctional elements regulating the permeability to solutes. These events result in a passive loss of fluids at the basis of diarrhea. Inhibition of protein phosphatases is also involved in other effects, such as the tumor-promoting properties of these toxins [1, 9, 10, 17].

Yessotoxin (YTX) group

Yessotoxin and its analogues are ladder-shaped polycyclic ethers, initially classified as DSP toxins, being detected with okadaic acid and its analogues. Subsequently, yessotoxins have been classified separately as they do not provoke diarrhoea. These compounds are produced by dinoflagellates, such as *Protoceratium reticulatum* (= *Gonyaulax grindley*), *Lingulodinium polyedrum* (= *Gonyaulax polyedra*) and *Gonyaulax spinifera*. Because these algal species are common in coastal waters of many regions, yessotoxins occurrence may be diffuse worldwide, and different filter feeding edible shellfish can accumulate these compounds. Anyway, there are no reports of human poisoning due to yessotoxins, whereas *in vivo* studies in mice a significant reduction in toxic potency after oral administration compared to intraperitoneal injection. On the other hand, *in vitro* studies evidenced cytotoxic effects for yessotoxin, even though its mechanism of action has not yet been clarified [18, 19].

Pectenotoxin (PTX) group

Pectenotoxins are polyether macrolides having a spiroketal group, three substituted oxolanes, a bicyclic ketal, and a six-membered cyclic hemiketal. They were initially classified as DSP toxins, being often detected in shellfish and phytoplankton together with these compounds. Nevertheless, the actual diarrhogenic potential of palytoxins and the actual health threat to consumers of contaminated mussels are unclear [20].

More than 20 pectenotoxin analogues have been identified in phytoplankton or shellfish from nany areas of the world, and several of them derive from a parent pectenotoxin metabolized by shellfish. The most commonly found pectenotoxin in algae is pectenotoxin-2, produced by dinoflagellates of the genus *Dinophysis*. Pectenotoxins are accumulated in the digestive glands of different filter-feeding bivalves, after filtration of *Dinophysis* cells [20, 21, 22]. These organisms can transfer the toxins to humans after their consumption, but no evidence of adverse effects is available [20, 23].

Palytoxins

Palytoxin (PLTX), originally isolated from the coral *Palythoa toxica* in Hawaii, is one of the most toxic nonprotein compounds known to date. It is a hydrophilic compound characterized by a long, partially unsaturated aliphatic polyhydroxylated backbone with spaced cyclic ethers, 64 chiral centers, and two amide groups. PLTX and a series of its analogs were subsequently identified in benthic dinoflagellates of the genus *Ostreopsis*, which are thought to be the producing organisms, although a bacterial origin has been suggested. *Ostreopsis* species bloom along tropical and subtropical coasts, but in the last years their distribution has increased in temperate seawaters, such as in the Mediterranean area [24, 25, 26].

PLTX is one of the most toxic natural compounds and its main molecular target seems to be Na^+/K^+-ATPase, a plasmalemmal pump involved in the maintenance of transmembrane ionic gradients in animal cells. PLTX binding to this pump converts it into a nonselective cationic channel, with consequent Na^+ influx into the cells and K^+ efflux, causing membrane depolarization and triggering adverse biological effects [27, 28, 29].

PLTXs can be accumulated in edible marine organisms, which ingestion can cause human poisonings with possible fatal outcomes. Human illness and death after consumption of crabs and fish contaminated or suspected to be contaminated with PLTXs have been reported in tropical and subtropical areas. The most frequent signs and symptoms ascribed to PLTX poisonings include gastrointestinal ones, myalgia, cramps, cardiac dysfunctions, dyspnoea and cyanosis. Nevertheless, the toxin identification and/or quantification in seafood are often incomplete or missing, and cases of human poisoning are frequently ascribed to these toxins only on the basis of symptoms, anamnesis and environmental/epidemiological data [30].

In the Mediterranean area, no human poisoning due to consumption of seafood contaminated by PLTX had been reported, so far. On the contrary, the increasing proliferation of *Ostreopsis* along the Mediterranean coastlines was accompanied by the occurrence of adverse effects in humans [31, 32, 33, 34]. In particular, human exposure to marine aerosol and/or seawater concomitantly to *Ostreopsis* proliferations were associated with an illness in which symptoms involved mainly the upper respiratory tract [32]. The cause-effect correlation between the cases of malaise and the involvement of algal toxins has not been completely clarified: in fact palytoxins were never detected in marine aerosol so far, even though these toxins were quantified in field algal samples [35].

Furthermore, although *Ostreopsis* cells concentrations were determined in seawater, these data are not predictive for human risk since dinoflagellates do not always produce the same amount of toxins, if any [36]. Anyway, the recurrence of sanitary problems associated with *Ostreopsis* blooms suggests a relationship between these phenomena [30]. Along the Italian coastlines, the first documented health problems associated with *Ostreopsis* blooms were described as general malaise in people exposed both to seawater and/or marine aerosol in Tuscany and Apulia [37, 38]. Later, symptoms such as rhinorrhea, cough, dyspnea and fever, associated with blooms along the Bari coast, were described accurately [31]. Similar symptoms were observed along the Spanish and French Mediterranean coasts, accompanied by ocular irritation, headache and, in some cases, by fever [33, 34]. Other anecdotal descriptions of respiratory problems following marine aerosol exposure during *Ostreopsis* blooms have also been reported along the Mediterranean coast [30]. The most serious sanitary problems occurred on the Liguria coast in summer of 2005 and repeated, with a lower intensity, in 2006 [32, 39]. In July 2005, more than 200 people enjoying the Genoa beach and promenade suffered an unusual influenza-like syndrome, characterized by a wide spectrum of symptoms such as fever, sore throat, cough, dyspnea, headache, nausea, rhinorrhea, lacrimation, vomiting and dermatitis. Approximately 20% of patients required hospitalization (1-3 days), and some of them needed the intensive care unit of the local hospitals [32]. This occurrence represents the most severe incident described to date in terms of both the number of people affected and for the severity of the symptoms. In addition to the problems at the respiratory level, skin irritation was frequently observed after aerosol exposure and/or seawater contact during *Ostreopsis* blooms. Indeed, in summer 2005, concomitant with marine aerosol exposure during *Ostreopsis* ovate blooms in Genoa the incidence of

dermatitis was 5% [32]. Erythematous dermatitis was also reported in patients exposed to marine aerosols during *Ostreopsis* blooms, along Apulia coasts [30]. Although the actual cause of this dermatitis has not yet been unequivocally determined, dermal toxicity has been associated to PLTX-like molecules contaminating other marine organisms [30, 40]. For instance, skin toxicity has been reported after handling zoanthids (*Palythoa*) used as aquarium decorative corals: persistent signs of dermotoxicity and perioral paresthesia were attributed to PLTX in a patient with intact skin [41]. Another case of skin toxicity due to handling PLTX-containing zoanthid corals (*Parazoanthus* sp.) involved a patient who cut his fingers while cleaning his aquarium. Dermal distress with swelling, paresthesia and numbness around the site of injury, as well as systemic symptoms, were recorded [30].

Cyclic imines group

Cyclic imine toxins are a group of macrocyclic compounds with imine- and spiro-linked moieties, including spirolides, gymnodimines, pinnatoxins, pteriatoxins, and prorocentrolides, produced by different species of dinoflagellates and accumulated in different shellfish species. Despite their toxicity observed in mice, no human poisoning syndrome due to these compounds has been documented [42].

RISK ASSESSMENT

A risk assessment process has to be carried out in order to ascertain the existence of any adverse effects on human health that may be caused by food additives and contaminants. The risk assessment process consists of four steps: first the hazard has to be identified, then characterized, then exposure assessed, and finally the risk itself characterized [43, 44].

The first step, hazard identification, entails identifying the type of adverse effects that can be triggered by a chemical in an organism, system or (sub) population [9]. Toxicity of dinoflaggelates can be caused by tiny quantities of toxins. As yet, no reasonable exposure limits have been set for biotoxins. The amount of toxicological data available on some toxins is very scarce and there is almost no information at all on chronic exposure to subsymptomatic doses.

The second step of characterizing the hazard entails carrying out dose-response assessment of the nature, relevance and mode of action of adverse effects produced by a chemical, in order to obtain quantitative data. Following appropriate animal testing the No Observable Adverse Effect Level (NOAEL, i.e. the highest tested doses at which no adverse effects are observed) can be established. This, in turn, can be used to set the Acute Reference Dose (ARfD) and the Tolerable Daily Intake (TDI) of a food contaminant. The ARfD is "the amount of a chemical in food that, in the light of present knowledge, can be consumed in the course of a day or at a single meal with no adverse effects" [9], and concerns the risk of acute effects subsequent to a single exposure to a substance. Acute toxicity studies in animals are often used to estimate the ARfD by applying a safety factor (also known as an uncertainty factor) to the NOAEL. It may also be possible to use data from exposure on a single day that is part of a repeat-dose study.

The default safety factor used has traditionally been 100, taking into account a 10-fold factor for variation in susceptibility between humans and experimental animals and a 10-fold factor to make an allowance for inter-individual variations in response among people, which also considers both the kinetics and target organ sensitivity of individuals. It is deemed that a 10-fold factor for human variability should be sufficient to include almost all individuals [45]. It may, in some cases, be necessary to use a different safety factor if, for example, toxicological data are of poor quality or if little information is available on the impact in man or on the adverse effects caused [46, 47].

The TDI is defined as "the daily intake of a chemical in food that, in the light of present knowledge, can be consumed every day for a lifetime with no appreciable harmful effects" [11, 48]. This relates to situations in which a chemical present in food may be consumed over very long periods, potentially with consequent chronic adverse effects on consumer health. The TDI is based on the NOAEL established in a range of both *in vivo* and *in vitro* studies. These include short-term toxicity (repeated daily doses for 14–28 days [49], sub-chronic toxicity (repeated daily doses for 90 days [50], chronic toxicity and carcinogenicity studies in animals [51, 52, 53], investigation of genotoxicity *in vitro* and *in vivo* [54], and reproductive and developmental toxicity. In the latter, animals are treated with repeat doses of the test substance prior to, during and following gestation [55, 56], possibly as part of a sub-chronic study [57]. It is also essential to have data on the absorption, distribution, metabolism and excretion of the substance. The risk assessment should be able to draw on information concerning the biochemical mechanism used by a toxic chemical exerts its adverse effects, which may help to identify target tissue of the chemical, in addition to identifying certain groups of individuals that may be more susceptible to its adverse effects. Hazard characterization of toxins should be based on

animal studies conducted using the administration route appropriate to humans. Therefore, logically, the oral route would be used when dealing with food additives and contaminants. Exposure assessment entails establishing how much of the chemical is consumed by humans. Risk characterisation consists of a combination of hazard identification, hazard characterization and exposure assessment in order to ascertain the degree of risk posed by a contaminant poses and the amount that would not be likely to cause adverse effects in humans. Shellfish toxins are particularly difficult to assess since the shellfish may contain a parent compound as well as many of its analogs, all belonging to the same group of toxins. Another issue adding to the previous difficulty is that some toxins are metabolized after consumption by predators, giving rise to other substances that may also be toxic [21, 58, 59, 60]. The risk assessment should assess the amount toxicity corresponding to each of the component toxins that contribute to the overall toxicity and set toxicity equivalence factors. The situation, which is already complicated, is aggravated by the fact that not only one class of toxin may contaminate the shellfish, and there may be cumulative or other effects of one class on another.

This makes for a daunting mission with the result that risk assessments related to biotoxins contaminating shellfish are often carried out using hazard characterizations based on incomplete data, very frequently with ArfDs calculated on acute toxicity data not reflecting the human situation, but from animals treated by intraperitoneal injection. It is rare for exposure estimates to be calculated using consumption and contamination data collected as part of the same event, possibly because of diagnostic and reporting issues.

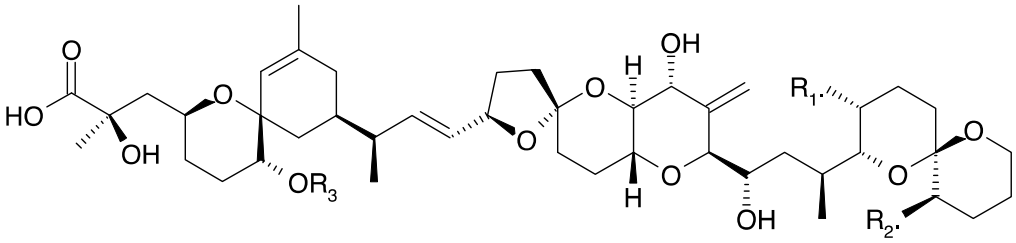
The toxicity in humans for saxitoxins, brevetoxins, azaspiracids, ciguatoxins, palytoxin and derivatives, okadaic acid and derivatives, tetrodotoxin and domoic acid has

been documented. Conversely, the acute toxicity of cyclic imines, yessotoxins and pectenotoxins demonstrated in animals has not yet been evidenced in humans [9].

In the following sections, informations for the risk assessment and legal aspects related to seafood contamination by okadaic acid-group toxins and palytoxins, an emerging group of toxins in Mediterranean Sea that can contaminate seafood together with okadaic acid and/or its derivatives, are reported.

OKADAIC ACID-GROUP TOXINS

Okadaic acid (OA)-group toxins are lipid-soluble and heat-stable polyethers including okadaic acid and its derivatives dinophysistoxins (DTXs), the most frequent being DTX-1, DTX-2 and DTX-3 (Figure 1).



	R_1	R_2	R_3
OA	**CH_3**	**H**	**H**
DTX-1	**CH_3**	**CH_3**	**H**
DTX-2	**H**	**CH_3**	**H**
DTX-3	**H or CH_3**	**H or CH_3**	**Acyl**

Figure 1: Chemical structure of okadaic acid and dinophysystoxin-1, -2 and -3.

Toxicity in animals

A series of toxicological studies on okadaic acid and its analogues dinophysistoxins had been performed in rodents after single oral or intraperitoneal administration or after repeated oral administration.

Single administration. After single *intraperitoneal injection* of okadaic acid to mice, lethalities were recorded at doses lower than 1 mg/kg, with LD_{50} (lethal dose for 50 % or the treated animals) ranging from 192 to 225 µg/kg [61]. Considering its analogues, an LD_{50} of 352 µg/kg was determined for dinophysistoxin-2 (DTX-2), estimating a potency

0.6 times lower than that of okadaic acid, ascribed to the axial 35-methyl group of DTX-2 that would reduce the toxin affinity for its molecular target, protein phosphatase 2A [62, 64]. Furthermore, intraperitoneal injection of OA or dinophysistoxin-1 (DTX-1) to rodents (mice and rats), at doses equal or higher than 200 μg/kg and 375 μg/kg, was shown to induce a rapid damage to the small intestine, particularly the mucosa of duodenum and upper jejunum. The authors observed three consecutive stages of intestinal injuy: (i) increased capillary permeability and exudation of serum into the lamina propria of intestinal villi; (ii) degeneration of absorptive epithelium; (iii) epithelial desquamation from the lamina propria. These alterations were reversible at sublethal doses and the recovery process was complete within 24 h [64, 65, 66]. DTX-3 induced less marked alterations, evidenced as dilation of the cysternae of Golgi apparatus and vesicles in the cytoplasm of the absorptive epithelium [64]. Intraperitoneal injection of these toxins to rodents induced also alterations in the liver, visible as vacuolisation and/or necrosis of hepatocytes [62, 64, 66, 67]. A liver damage was observed also after *intravenous OA injection* to rats as congestion and dissolution of bile canalicular actins sheaths [68].

After single *oral administration*, OA, DTX-1 and DTX-3 were shown to be less toxic than intraperitoneally: after *per os* administration, the LD_{50} of OA in mice ranges between 1 and 2 mg/kg [67]. The signs of toxicity, which include diarrhoea, are similar to those observed after intraperitoneal injection. DTX-1 and DTX-3 were shown to cause also the degeneration of gastric mucosa cells [64, 66, 68, 69].

Repeated administration. A study after daily oral OA administration to mice (1 mg/kg/day, for 7 days) showed diarrhoea induction, body weight loss, reduced food consumption, and the death of 2/5 mice at day 5 of administration. Tosic effects were

noted at non-glandular stomach and liver, beaides ultrastructural changes at mitochondria and fibrillar structures of cardiomyocytes [70].

Tumour promoting activity. Okadaic acid and DTX-1 are tumour promoters, as observed by two-stage carcinogenesis studies in rat glandular stomach [71] and mouse skin [72].

Absorption, distribution, excretion and metabolism

Kinetic studies on okadaic acid are limited and a study in mice evidenced that 24 h after single oral [^{3}H]-OA administration (50 or 90 μg/kg), the toxin was detectable in all organs and fluids, including skin, but was more concentrated in urine, intestinal content and intestinal tissues. OA was slowly eliminated, which suggested an enterohepatic circulation [73]. Subsequently, Ito et al. [74] revealed that after oral administration of OA (75-250 μg/kg) to mice, the toxin was rapidly absorbed, mainly from jejunum, and reached the liver within 5 min. An immunostaining method, showed that the toxin was distributed in almost the whole body, andat intestinal level was detectable also after two weeks. Excretion, from kidneys and intestine, began 5 min after administration, and the toxin was detectable in feces also after 4 weeks. Another study after acute oral OA administration in mice (115 and 230 μg/kg) revealed low amounts of toxin in the liver after 24 and 48 h, respectively, whereas the toxin was detected in the duodenum and ileum but not in the colon secretions [75]. In general, after acute oral administration in mice, distribution of OA was: intestinal content > urine > feces > intestine tissue > lung > liver > stomach > kidney > blood [76].

Okadaic acid given orally to pregnant mice at day 11 of gestation passed to the foetuses; the study did not, however, include observations of possible effects on the offspring [77].

In humans, a poisoning episodecaussed by the ingestion of mussles contaminated by DTX-3 gave evidence for a biotransformation of the toxin into DTX-1, which was the only DSP toxin detected in the faeces of intoxicated subjects [78].

Human epidemiological data

The consumption of shellfish contaminated by OA and/or its analogues cause a diarrheic syndrome (DSP), which signs and symptoms are mainly diarrhea, nausea, vomiting and abdominal pain. Although the poisoning can be debilitating for some days, no fatal cases from DSP were reported, so far, and recovery is usually complete within 3 days [15].

Since the discovery of DSP toxins in '70, DSP outbreacks had been reported worldwide. Nevertheless, documented episodes including an exposure estimate (consumption and contamination data collected simultaneously) are still limited. This may be due to underdiagnosis and/or lack of dpocumentation. In fact, consumers suffering from mild gastrointestinal disturbances do not consult a physician and, when they do it, physicians might not diagnose DSP as gastrointestinalk symptoms are unspecific [79].

Mechanism of action

Okadaic acid and DTX-1 are inhibitors of protein phosphatases, mainly protein phosphatases 1 and 2A, enzymes involved in the dephosphorylation of serine/threonine protein residues in eukaryotic cells [80]. These enzymes inhibition leads to a rapid increased level of phosphorylated proteins cells. Also diarrhea and alterations of small intestinal absorptive epithelium induced by these compounds have been attributed to an increased level of the phosphorylation in proteins controlling ions secretion in intestinal cells as well as of cytoskeletal and/or junctional elements regulating the permeability to solutes. In fact, these events result in a passive loss of fluids at the basis of diarrhoea [22].

Inhibition of protein phosphatases is also involved in other cellular effects, including the tumor-promoting properties of these toxins [72]. A free carboxyl group in the DSP molecule is essential for protein phosphatase inhibition, since methyl and diol esters do not show inhibition [81].

Legislation and risk assessment

Regarding the control of OA and its analogues in seafood, the European Union regulation establishes that commercialized molluscs must not contain OA and its derivatives in total quantitiesthat exceed the limit of 160 μg OA equivalents/kg edible parts [82].

For the risk characterization, which combines information on types of adverse effects (hazard identification) and exposure assessments to estimate the probability and severity of adverse health effects, EFSA's opinion on OA group toxins led to establish a limit for the total amount of biotoxins that can be ingested within 24 h without significant health risk. In particular, on the basis of available human data, a Lowest Observed Adverse Effect Level (LOAEL; 50 μg OA equivalents/person, corresponding to 0.8 μg OA equivalents/kg body weight of an adult of 60 kg) was considered. Using an uncertainty factor of 3, a No Observed Adverse Effect Level (NOAEL) was estrapolated an then an acute reference dose (ARfD, an estimate of a daily oral exposure for an acute duration - 24 h or less - to the human population that is likely to be without an appreciable risk of deleterious effects during a lifetime) of 0.3 μg OA equivalents/kg body weight was calclated. This was then used to set a limit based on an approximate quantity of shellfish consumption, i.e. 45 μg OA equivalents/kg shellfish flesh, vs. the European Union directive of 160 μg OA equivalents/kg shellfish flesh [82].

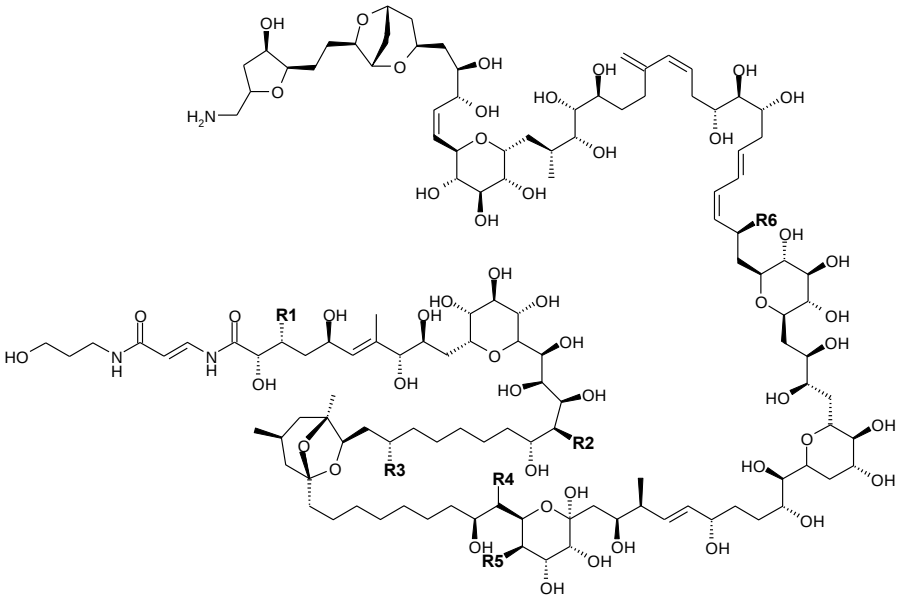
EFSA concluded that an assessment of human exposure to DSP toxins can not be performed due to the lack of representative data [17]. Anyway, for the risk characterization, on the basis of the available consumption and occurrence information, EFSA considered that there is a chance of 20% of exceeding the ARfD (0.3 μg OA equivalents/kg). EFSA's opinion is that "DSP occurs under the current legislation and the prescribed methods of control", which was the mouse bioassay, based on the observation of survival of mice after an intraperitoneal injection of a shellfish extract [17]. However, a new method for control of lipophilic toxins (liquid chromatography associated to tandem mass spectrometry; LC-MS/MS) has been then approved to substitute the mouse bioassay for DSP toxin detection in shellfish [83]. Regular checks must be carried out to detect any toxin-producing plankton in production and relaying waters, and any biotoxins in live bivalve molluscs. These checks should normally be weekly during the periods in which harvesting is allowed, but could be more or less frequent depending on the risk anticipated in each particular area.

PALYTOXINS

Palytoxin (PLTX) is a water-soluble compound characterized by a long, partially unsaturated aliphatic polyhydroxylated backbone with spaced cyclic ethers, 64 chiral centers and two amide groups (Figure 2). PLTX and a series of its analogues, such as homo-PLTX, bishomo-PLTX, neo-PLTX, deoxy-PLTX and 42-hydroxy-PLTX (Figure 2) were identified in *Palythoa* species. In addition, PLTX and its analogues, including ostreocin-D (Figure 2), mascarenotoxins and ovatoxins, were identified in benthic dinoflagellates of the genus *Ostreopsis*, which have been proposed as producing organisms even though a bacterial origin has been suggested [24, 25].

Toxicity in animals

Single administration. The acute toxicity of palytoxin is strongly dependent upon the exposure route, being high after *parenteral administration*. Also the animal species seem to be differentially susceptible to this toxin. Rabbits, dogs, rhesus monkeys and rats seem to be the most susceptible species to intravenous injection of PLTX, and the mouse the least susceptible, although variations in LD_{50} values (0.025-0.53 μg/kg) were recorded in mice. These differences could be due to the fact that different toxin preparations, with unknown purity, have been used, mainly in the first studies when the reported molecular weight of PLTX was higher than hat actually known. PLTX is less toxic intraperitoneally than by intravenous injection, and LD_{50} values in mice range from 0.31 to 1.5 μg/kg. The intraperitoneal toxicity in mice of the analogue ostreocin-D was lower than that of PLTX (lethality > 4 μg/kg). LD_{50} of PLTX after intraperitoneal injection is similar to that recorded after intramuscular or subcutaneous injections. Palytoxins were observed to be highly toxic also by intratracheal instillation: the lethal dose of PLTX and ostreocin-D was > 2 μg/kg and 11-13 μg/kg, respectively [84, 85].



	R1	R2	R3	R4	R5	R6
Palytoxin	CH_3	OH	CH_3	H	OH	OH
42-hydroxy-palytoxin	CH_3	OH	CH_3	OH	OH	OH
Ostreocin-D	H	H	H	OH	H	OH

Figure 2: **Chemical structure of palytoxin and of two of its analogues.**

Following intraperitoneal injection of PLTX to mice, adhesions were observed in the peritoneum, with ascities and small intestine dilation. Histologicl analysis showed the presence of single-cell necrosis in the heart as well as necrosis of thymus and spleen lymphocytes. Bleeding, oedema and necrosis were observed in the small intestine. Ultrastructural analysis by electron microscopy showed also rounding of mitochondria and separation of organelles in myocytes, loss microvilli in renal tubules and vacuolation

of pancreatic acinar cells [97, 98]. Injection of ostreocin-D to mice caused erosion in the stomach and intestines [85].

Palytoxins are much less toxic after *oral administration* and LD_{50} values in mice were calculated in the range 510-767 μg/kg for PLTX [27, 85], comparable to that of 42-hydroxy-PLTX (LD_{50} = 651 μg/kg) [86]. No lethality was observed for ostreocin-D at 300 μg/kg [87]. PLTX and ostreocin-D (200 μg/kg) seem to be toxic also after *sublingual administration* to mice [87].

After oral PLTXadministration, increased plasma levels of creatine phosphokinase, lactate dehydrogenase and aspartate transaminase were recorded in mice. At the histological level, non-glandular stomach inflammation was observed in mice surviving up to 24 h after administration. Other tissue alterations were noted in the liver and pancreas, while cardiac and skeletal muscle cells revealed only ultrastructural alterations, visible as aggregates of mitochondria and fibrillar alterations [85]. Similar findings, accompanied by increased plasma levels of potassium ions, were noted in mice after oral administration of 42-hydroxy-PLTX [86].

Intratracheal instillation of PLTX to mice caused alveolar haemorrhage, pulmonary oedema, gastrointestinal erosion and glomerular atrophy. Similar lung injuries were observed in ostreocin-d treated mice but showed slower progression and recovery than those from PLTX [87].

Repeated administration. Studies describing the effects of repeated PLTX administration deserve special consideration because they allow the identification of target organs in a situation mimicking a characteristic type of human exposure. After repeated *intraperitoneal injections* of PLTX to mice (0.25 μg/kg, 5 times/week: 5, 10, 15 and 29 total injections), 29 doses of the toxin induced diarrhoea and peritonitis (60 % of

animals) as well as necrosis of thymic and splenic lymphocytes. Decreased thymus weight and increased spleen weight were noted after 10-15 doses. After 29 injections, these changes were reversible within one month [88].

After repeated *sublingual administration* of PLTX (up to 3 days, cumulative dose up to 495 μg/kg) or ostreocin-D (up to 5 days of treatment, cumulative dose up to 1000 μg/kg), pulmonary congestion and mild alveolar destruction accompanied by stomach ulcers and gut erosions were observed. Mice treated with PLTX showed more severe alterations than those treated with ostreocin-D [87].

Tumour promoting activity. The tumor promoting activity of palytoxin was evidenced by two-stage carcinogenesis studies in mouse skin: cutaneous application of PLTX (0.5 μg two times per week for 30 weeks) after initiation with 7,12-dimethylbenz[a]antracene resulted in the development of tumors in 62.5% of the treated animals [89]. Tumor promoting activity was confirmed *in vitro* by a two-stage transformation assay using Balb/c 3T3 cells: treatment with 1.9 pM PLTX increased the number of transformed foci after initiation by 3-methylcholanthrene [90]. The tumor promoting activity seems to be mediated by stimulation of mitogen activated protein (MAP) kinases, a family of serine/threonine kinases that relay a variety of signals to the cellular machinery that regulates cell fate and functions [91].

Absorption, distribution, excretion and metabolism

No studies on kinetic of palytoxins are available.

Human epidemiological data

In general, exposure routes to PLTXs may be by: (i) ingestion of contaminated seafood; (ii) skin exposure to seawater or corals containing PLTXs; (iii) inhalational exposure to aerosolized seawater during *Ostreopsis* blooms or water from aquaria

containing contaminated corals during their mainteinance; or (iv) ocular exposure so seawater. The main concern related to the entrance of palytoxin into the ecosystem is the potential human exposure through the consumption of contaminated seafood. In fact, PLTX and/or PLTX-like compounds have been associated to cases of seafood poisonings In the majority of cases, PLTXs were hypothesized as a causative agents of poisoning on the basis of clinical symptoms and case history, in particular when PLTX-producing and/or containing organisms were involved [30].

Oral intoxications by PLTXs, with lethal outcomes, have been reported from tropical and subtropical regions after the ingestion of contaminated fish and crubs [92, 93, 94, 95]. These main signs of toxicity reported during these intoxications included initial gastrointestinal symptoms, typically nausea, vomiting and diarrhea as well as myalgia, muscle cramps, cardiac dysfunctions, respiratory problems, and cyanosis, with frequent elevated serum levels of creatine phospholinase and myoglobinuaria raised by possible toxic effects at muscular level [92, 93, 94, 95]. In lethal cases, convulsions and delirium were also reported before death [93, 94].

Other cases of poisoning attributed to PLTXs are reported but quantification of toxin was not performed in the leftovers. They are related to ingestion of potentially PLTXs-contaminated seafood and the documented signs and symptoms were similar to those previously described [30].

Mechanism of action

Palytoxin binds to the plasmalemmal Na^+/K^+ ATPase, transforming it into a nonselective channel permeble to monovalent cations. The Na^+/K^+ ATPase is a transmembrane pump belonging to the family of P-type ATPases, which are essential for maintaining cellular homeostasis: it transfer three Na^+ ions out of the cell in trade for two

K^+ ions into the cell in a cyclic process that exploits the hydrolysis of ATP. Therefore, after PLTX binding, the gates on the two sides of the membrane are simultaneously opened with consequent Na^+ influx into the cells and K^+ efflux, causing membrane depolarization in excitable and non excitable cells and triggering a series of adverse biological effects [28, 29].

Legislation and risk assessment

Despite the increasing occurrence of *Ostreopsis* species producing palytoxins also in temperate coastal areas, as in Mediterranean Sea, there are no regulations on PLTXs in commercialized seafood neither in European Union nor in other countries. Considering the lack of reliable quantitative data on PLTXs toxicity in humans and the LOAEL of 200 μg PLTX/kg for acute oral toxicity in mice as the reference point, the CONTAM Panel of the European Food Safety Authority expressed its opinion on palytoxins toxicity. EFSA derived an ARfD of 0.2 μg/kg body weight, applied to the sum of palytoxin and ostreocin-D, using the uncertainty factors of 10 in addition to the default uncertainty factors of 10 for intra- and interspecies variations. On the basis of the derived ARfD, the maximum concentration of palytoxins (sum of PLTX and ostreocin-D) in shellfish was calculated to be 30 μg/kg shellfish meat, considering 400 g of seafood consumption by a person of 60 kg body weight [96].

CONTAM Panel of EFSA could not characterize the risk related to exposure to PLTXs due to the lack of data on exposure assessment [96].

COMBINATION OF OKADAIC ACID AND PALYTOXIN

Okadaic acid was frequently detected in musslels and the increasing proliferation of dinoflagellates of the genus *Ostreopsis* that can potentially produce palytoxins, induce to consider a possible seafood contamination by both these toxins and a consequent human co-exposure by its consumption. Thus, studies aimed to characterize the toxicity by oral co-exposure to okadaic acid and palytoxin are needed.

PURPOSE OF THE STUDY

The increasing distribution of microalgae which may produce palytoxins or okadaic acid poses concern on possible accumulation and co-occurrence of these toxins in seafood, which consumption could lead to additive/synergistic toxic effects. Thus, the aim of the present study was to evaluate the acute and short-term oral toxicity of combined doses of palytoxin and okadaic acid in mice, in comparison to each toxin alone. In particular, the purpose of the present study was to evaluate the maximum and feasible tolerability and identify the target organs of PLTX and OA when administered alone or in combination to give a contribution in the risk characterization of the two toxins as seafood contaminants.

MATERIALS AND METHODS

Toxins and other materials

Okadaic acid (OA; purity: 98 %) and palytoxin (PLTX; purity: higher than 90 %) were purchased from Wako Pure Chemical Industries (Osaka, Japan). If not otherwise indicated, other chemicals, of analytical grade, were purchased from Sigma aldrich (Milan, Italy).

Animals and experimental conditions

Female ICR (CD-1) mice (18-20 g body weight, 4 weeks old) were purchased from Harlan Laboratories (S. Pietro al Natisone, Udine, Italy).

The original group of Swiss mice that served as progenitors of this stock consisted of two male and seven female albino mice derived from a non-inbred stock in the laboratory of Dr. de Coulon, Centre Anticancereux Romand, Lausanne, Switzerland. These animals were imported into the United States by Dr. Clara Lynch of the Rockefeller Institute in 1926. The Hauschka Ha/ICR stock was initiated in 1948 at the Institute for Cancer Research (ICR) in Philadelphia from “Swiss” mice of Rockefeller origin. To Dr. Edward Mirand of Roswell Park Memorial Institute where they were designated as HaM/ICR. To Charles River in 1959.

Animals were acclimatized for one week before the experiments. Controlled temperature (21 ± 1° C) and humidity (60-70 %) were maintained in the animal room, illuminated with a fixed artificial light cycle (7.00 a.m.–7.00 p.m.). Animals were kept in polycarbonate cages, using dust free poplar chips for bedding and fed with the standard diet for rodents (Harlan Laboratories; S. Pietro al Natisone, Udine, Italy). Diet composition, as indicated by the supplier, was: proteins (18.5 %), fats (5.5 %), fibers (4.5

%), hashes (6.0 %), non-nitrogen compounds (53.5 %), water (12.0 %). Water and feed were provided ad libitum during the entire duration of the experiments.

Animal experiments were carried out at the University of Trieste, Italy in compliance with the Italian Decree n. 116/1992 as well as the EU Directive 2010/63/EU and the European Convention ETS 123.

ACUTE ORAL TOXICTY OF PALYTOXIN AND OKADIAC ACID COMBINATION

The doses of okadaic acid and palytoxin were selected on the basis of previous studies in mice after 7-day oral administration. Okadaic acid dose was selected from an exploratory repeat dose study (data not shown) where female CD-1 mice were 7-day treated with a range of doses (750, 370 or 185 μg/kg/day). The dose of 370 μg/kg was identified as the maximum tolerated one: clinical signs were reversible on a short term (days) and diarrhoea was not debilitating the animals (data not shown). The doses of palytoxin were selected considering that the lowest lethal acute oral dose in CD-1 mice was 600 μg/kg [85137] , while the daily repeated oral administration induced toxicity signs at 30 μg/kg/day, starting from the third day of treatment. OA was administered at 370 μg/kg, while PLTX doses were 30, 90 and 270 μg/kg (Table 1). The oral route is the route by which intoxications generally occur and by which the toxins elicited their preselected behaviour. Oral gavage was used as administration method instead of the 'contaminated food' in order to have certainty of the administered doses within the same animal and the same dose groups.

Animals were fasted for 3 h and weighed immediately before treatment (9.00-10.00 a.m.). Groups of 8 mice were administered with single oral doses of PLTX (30, 90 or 270 μg/kg), OA (370 μg/kg) or combined doses of PLTX and OA (30 and 370, 90 and 370 or 270 and 370 μg/kg, respectively). Control mice were administered with the vehicle alone (10 ml/kg of phosphate buffered saline, PBS, containing 1.8 % ethanol, v/v). Toxins, or vehicle, were administered by gavage at 10 ml/kg, adjusted on the basis of the recorded body weight. After dosing, feed was returned within 2 h and, during the observation period, it was offered ad libitum. A subgroup of 5 mice was sacrificed and a second

subgroup of 3 mice was maintained for a 14-day withdrawal period. Clinical signs and symptoms, body weight and food consumption were evaluated daily in the morning. Mortality was checked twice daily. At the scheduled sacrifice (24 h or 14 days after the treatment), animals were anesthetized with ketamine hydrochloride (350 mg/kg; Inoketam100; Virbac; Milan, Italy) and bled to death through the abdominal aorta to collect blood for hematochemical analysis (see Section 2.4). Mice were necropsied and liver, heart, lungs, kidney, spleen and brain were removed and weighed. The main organs and tissues (see Section 2.5) were fixed in neutral buffered formalin for the histological analysis. Similarly, animals which died during the experiment were immediately weighed and the blood was collected for clinical pathology analysis; the main organs and tissues were weighed and/or fixed for the histological evaluation, as reported above.

The day of the treatment is referred to as day 1 of the study.

Table 1: **Acute oral toxicity of combined oral doses of palytoxin and okadaic acid: experimental scheme.**

Group (No. of animals)	Doses of PLTX (µg/kg)	Doses of OA (µg/kg)	Scheduled sacrifice at 24 h: number of mice	Scheduled sacrifice after 14 days: number of mice
1 (16F)	0	0	10	6
2 (16F)	0	370	10	6
3 (8F)	30	0	5	3
4 (3F)	90	0	5	3
5 (8F)	270	0	5	3
6 (8F)	30	370	5	3
7 (8F)	90	370	5	3
8 (8F)	270	370	5	3

TOXICITY OF PALYTOXIN AFTER REPEATED DOSE ORAL ADMINISTRATION

Two repeated dose experiments were carried out: an initial experiment (Experiment 1) identified PLTX toxic doses following repeated administration and Experiment 2 was designed in order to identify a no effect level following repeated administration.

Experiment 1

Groups of 8 mice were treated, once a day for 7 days (days 1-7), at three PLTX doses (30, 90 or 180 μg/kg/day) or vehicle (PBS; 10 ml/kg/day containing 1.8 % ethanol, v/v). PLTX was dissolved in PBS, pH 7.0: to avoid chemical instability, solutions were prepared immediately before administration (9.00-10.00 a.m.). Animals were fasted for 3 h and weighed immediately before treatment (9.00-10.00 a.m.). After dosing, feed was returned within 2 h and, during the observation period, it was offered ad libitum. A subgroup of 5 mice was sacrificed and a second subgroup of 3 mice was maintained for a 14-day withdrawal period in order to asses potential effects of a n off treatment period. Clinical signs and symptoms (every 12 hours), body weight and food consumption were evaluated daily in the morning.Moratlity was checked twice daily. At the scheduled sacrifice (24 h or 14 days after the treatment), animals were anesthetized with ketamine hydrochloride (350 mg/kg; Inoketam100; Virbac; Milan, Italy) and bled to death through the abdominal aorta to collect blood for clinical pathology (see Section 2.4). Mice were necropsied and liver, heart, lungs, kidney, spleen and brain were removed and weighed. The main organs and tissues (see Section 2.5) were fixed in neutral buffered 10% formalin for the histological analysis. Similarly, animals which died during the experiment were immediately weighed and the blood was collected for clinical pathology

analysis; the main organs and tissues were weighed and/or fixed for the histological evaluation, as reported above.

The day of the first treatment was referred to as day 1 of the study. The experimental scheme is reported in Table 2.

Experiment 2

Experiment 2 mimicked the experimental design and investigated in detail the results obtained by Experiment 1 in order to identify a dose with no effect, which had not been identified by the previous (see Results section).

Groups of 8 mice were treated once a day for seven days with palytoxin (3 or 30 μg/kg/day) or vehicle (PBS; 10 ml/kg/day). Animals were fasted for 3 h and weighed immediately before treatment (9.00-10.00 a.m.). Doses were estimated on the mornings' body weight. After dosing, food was restored within 2 h and, during the observation period, it was offered ad libitum. A subgroup of 5 mice was sacrificed and a second subgroup of 3 mice was maintained for a 14-day withdrawal period in order to asses potential effects of an off treatment period. Clinical signs and symptoms (every 12 hours), body weight and food consumption were evaluated daily in the morning. Mortality was checked for twice a day. At the scheduled sacrifice (24 h or 14 days after the treatment), animals were anesthetized with ketamine hydrochloride (350 mg/kg; Inoketam100; Virbac; Milan, Italy) and bled to death through the abdominal aorta to collect blood for clinical pathology (see Section 2.4). Mice were necropsied and liver, heart, lungs, kidney, spleen and brain were removed and weighed. The main organs and tissues (see Section 2.5) were fixed in neutral buffered 10% formalin for the histological analysis. Similarly, animals which died during the experiment were immediately weighed and the blood was

collected for clinical pathology analysis; the main organs and tissues were weighed and/or fixed for the histological evaluation, as reported above.

The day of the first treatment was referred to as day 1 of the study. The experimental scheme is reported in Table 2.

Table 2: **Toxicity of palytoxin after repeated oral administration: experimental scheme.**

Group (No. of animals)	Doses of PLTX (µg/kg/day)	Number of animals sacrificed on day 8	Number of animas sacrificed after 14 d of recovery
First experiment			
1 (6F)	0	3	3
2 (6F)	30	3	3
3 (6F)	90	3	3
4 (6F)	180	3	3
Second experiment			
1 (6F)	0	5	3
2 (6F)	3	5	3
3 (6F)	30	5	3

TOXICITY OF PALYTOXIN AND OKADAIC ACID ASSOCIATION AFTER REPEATED ORAL ADMINISTRATION

A non diarrhoeic dose of OA (185 μg/kg/day) was selected from a previous exploratory repeat dose study where female mice were treated with oral doses of the toxin for 7 days (750, 375 or 185 μg/kg/day) and only the lowest dose did not induce diarrhea. PLTX was administered at two doses: 3 μg/kg/day, the NOAEL determined by the previous toxicity study with PLTX after repeated daily administration in mice, and 10 μg/kg/day, a dose intermediate between the NOAEL and the LOAEL.

Animals were fasted for 3 h and weighed immediately before treatment (9.00-10.00 a.m.). After dosing, feed was returned within 2 h and, during the observation period, it was offered ad libitum. A subgroup of 5 mice was sacrificed and a second subgroup of 3 mice was maintained for a 14-day withdrawal period in order to asses potential effects of an off treatment period. Clinical signs and symptoms (every 12 hours), body weight and food consumption were evaluated daily in the morning. Mortality was checked twice daily. At the scheduled sacrifice (24 h or 14 days after the treatment), animals were anesthetized with ketamine hydrochloride (350 mg/kg; Inoketam100; Virbac; Milan, Italy) and bled to death through the abdominal aorta to collect blood for clinical pathology (see Section 2.4). Mice were necropsied and liver, heart, lungs, kidney, spleen and brain were removed and weighed. The main organs and tissues (see Section 2.5) were fixed in neutral buffered 10% formalin for the histological analysis. Similarly, animals which died during the experiment were immediately weighed and the blood was collected for clinical pathology analysis; the main organs and tissues were weighed and/or fixed for the histological evaluation, as reported above.

The day of the first treatment was referred to as day 1 of the study. The experimental scheme is reported in Table 3.

Table 3: **Toxicity of palytoxin and okadaic acid after repeated oral administration: experimental scheme.**

Group (No. of animals)	Doses of PLTX (µg/kg)	Dose OA (µg/kg)	Number of animals sacrificed on day 8	Number of animas sacrificed after 14 d of recovery
1 (8F)	0	0	5	3
2 (8F)	3	185	5	3
3 (8F)	10	185	5	3
4 (8F)	3	-	5	3
5 (8F)	10	-	5	3
6 (8F)	-	185	5	3

DESCRIPTION OF OBSERVATIONS FOR CLINICAL SIGNS

Physical appearance, behavior and general and local clinical signs were observed daily (paralysis, tremor activity, abnormal gait, cyanosis, diarrehoea, muscle tone, writhing reflex, sedation, reactivity to touch, piloerection, abdominal dilation, hypersensitivity to light).

Any deviation from normal physiological value was scored using a severity scale in which the minus (-) sign corresponded to a decrease of the parameter observed (when applicable) and a positive sign (+), an increase occurrence of the parameter.

CLINICAL CHEMISTRY

Biochemistry was performed at each scheduled sacrifice time point. In animals which died prematurely, blood was collected for sampling, where possible.

Clinical chemistry was conducted on serum obtained from whole blood collected from the abdominal aorta at necropsy. Whole blood collected in SST tubes was allowed to clot for about 30-60 minutes at room temperature. Blood was centrifuged at 2200 x g for 10 min and serum was transferred to the analysis tube.

Analyses were conducted using an automatized blood analyzer AU400 Olympus with dedicated reagents (Beckman Coulter, except for GLDH reagent, supplied by Diasys). The following parameters were evaluated:

Table 4: Clinical chemistry parameters evaluated

Parameters	Units	Methods
Glucose	mg/dL	Hexokinase
Total Cholesterol	mg/dL	CHOD-PAP (enzymatic)
Triglycerides	mg/dL	GPO-PAP (enzymatic)
Urea	mg/dL	Urease-glutamatic DH
Creatinine	mg/dL	Jaffé kinetic without deproteinization
Aspartate aminotransferase (AST)	U/L	IFCC without P5P
Alanine aminotransferase (ALT)	U/L	IFCC without P5P
Alkaline phosphatase (ALP)	U/L	IFCC
Glutamate dehydrogenase (GLDH)	U/L	DGKC
Total bilirubin	mg/dL	DPD
Total protein	g/dL	Biuret
Albumin	g/dL	Bromcresol green
Globulin	g/dL	Calculated
Albumin/globulin ratio	-	Calculated
Sodium	mEq/L	ISE, indirect
Potassium	mEq/L	ISE, indirect
Chloride	mEq/L	ISE, indirect
Calcium	mg/dL	oCPC
Inorganic phosphorus	mg/dL	Molybdate UV

GROSS PATHOLOGY

Necropsy and examination for gross pathological alterations was performed on all mice including those that are killed in extremis or die in the course of the study on selected organs. The surviving mice were sacrificed by anesthesia with an intra-peritoneal injection of an overdose of sodium pentothal (at least 150 mg/kg). After the blood sampling, selected organ were removed and stored in buffered formalin for histological analyses. Histological analyses were conducted by optical microscope.

Table 5: Selected organs sampled for histological purposes.

Liver (all lobes)	Heart
Lungs	Spleen
Thymus	Kidneys
Pancreas	Schelectric muscle
Stomach (glandular and non-glandualar)	Digiunum
Duodenus	Colon
Rectum	Brain
Cervelletto	Spinal cord
Uterus	Ovarius

TISSUE FIXATION AND INCLUSION

The formalin is considered the best fixative generic, since preserves most of the cellular structures, requires a short time of fixation, can be used for a long period of conservation and penetrates quickly without excessive hardening tissues. It neutralizes the basic groups increasing the acidity of the protein, so the formalin-fixed tissues have greater affinity for basic dyes and for those acids. It does not precipitate the DNA and proteins, retains most of the lipids and phospholipids making them insoluble in solvents, it does not fix the soluble carbohydrates and it solubilizes glycogen and urea. Tissue samples were left in the fixative for a minimum period of 48 hours in relation to the size of the tissues before being processed.

The organs / tissues were included in paraffin in order to facilitate the cutting, giving them a proper consistency for the follows steps. The inclusion in paraffin can be effected only after the elimination of the water contained in the tissues since the paraffin and water / aqueous solutions are not miscible. Therefore, the tissues were subjected to a dehydration process in order to removal water from the tissues through a progressive replacement of water with alcohols in increasing concentrations than replace the alcohol with xylene, and then with paraffin. In fact, the alcohols are miscible with water; xylene is miscible with alcohol and paraffin with xylol. The alcohol used in the stages of the process of inclusion is pure ethyl alcohol. The paraffin used has a nominal melting temperature (temperature specified by the manufacturer in the technical specifications of the product) between 56 and 58°C.

The table hereafter summarizes the steps required for tissue inclusion.

Table 6: **Tissue inclusion steps**

Step	Reagent	Time (h)
1	Ethyl alcohol 50%	1
2	Ethyl alcohol 70%	1
3	Ethyl alcohol 80%	1.50
4	Ethyl alcohol 96%	1.50
5	Ethyl alcohol 96%	1.50
6	Ethyl alcohol 99.9%	1.50
7	Ethyl alcohol 99.9%	1.50
8	Ethyl alcohol 99.9% + Xilol	1.50
9	Xilol	1
10	Xilol	1
11	Paraffin	1.50

All the steps described above were conducted with Leica EG1160 apparatus.

CUTTING

Before cutting procedure the tissue blocks paraffin-embedded were prepared by cooling in a refrigerator at a temperature of +5° C and subsequent cooling to a temperature of -7° C using the Cold Plate TEC 2900 Histo-Line.Cutting procedures were performed using a rotary microtome Leica RM2255.

The obtained sections with thickness of about 5 microns were placed in heated deionized water bath (about 40° C) to allow a greater distension of the slices and their easier collection. Prior to staining procedure the glass slides were left to dry in an oven at about 39° C for about 3 hours.

STAINING

The standard staining of paraffin-embedded tissues is hematoxylin and eosin. Hematoxylin is a basic dye causes the blue color of the nucleus while the eosin (acid) stain the cytoplasm in pink. Since the dyes are aqueous solution, the tissue sections

must be previously de-waxed starting with xylol than flow through a series of alcohol with descending gradation down to the water. Once staining was completed, sections were dehydrated again with passage of a series of alcohol with ascending gradation up to xilol. The staining procedures were conducted by automatic stainer Leica 5020. The following table describes the standard staining steps.

Table 7: Staining steps

Step	Reagent	Time (min)
1	Xilol	9
2	Xilol	5
3	Ethyl alcohol 99.9% + Xilol	1
4	Ethyl alcohol 99.9%	1
5	Ethyl alcohol 80%	1
6	Ethyl alcohol 50%	1
7	Deionised water	1
8	Deionised water	1
9	Hematoxylin	9
10	Water	15
11	Deionised water	1
12	Eosin	1
13	Eosin	1
14	Deionised water	1
15	Ethyl alcohol 70%	1
16	Ethyl alcohol 95%	1
17	Ethyl alcohol 95%	1
18	Ethyl alcohol 99.9%	1
19	Ethyl alcohol 99.9%	1
20	Ethyl alcohol 99.9% + Xilol	1
21	Xilol	2
22	Xilol	3

The procedure to obtain Hemotoxylin working solution was the follow:

Potassium alum	50 g
Potassium iodate	0.2 g
Glycerin extra-pure grade	200 mL
Deionised water	to 1 L

The procedure to obtain Eosin working solution was the follow:

Eosin	2 g
Glacial acetic acid	2.4 mL
Deionised water	to 1 L

After the staining procedure were completed the tissues slides were sealed with a second glass slide. This automatized procedure was conducted with Leica CV5000 apparatus.

RESULTS

ACUTE ORAL TOXICTY OF PALYTOXIN AND OKADAIC ACID COMBINATION

The toxic effects of combined doses of palytoxin and okadaic acid was firstly investigated after their acute oral administration in mice. To this aim, groups of 8 animals were administered by gavage with palytoxin (30, 90 or 270 μg/kg) in combination with 370 μg/kg of okadaic acid, which effects were compared to that of each toxin alone at the same doses.

Mortality and Clinical signs

No animals died in the group treated with the lowest dose of PLTX (30 μg/kg) in combination with OA (370 μg/kg). Lethal episodes were recorded in mice administered with the toxins blend starting with the combination of 90 μg PLTX/kg and 370 μg OA/kg. At this doses combination, 2/8 mice died within 3 h from administration. In the experimental group given 270 μg PLTX/kg and 370 μg OA/kg, death occurred in 3/8 mice: two mice died within 3 h and one on the fifth day following dosing. Administration of PLTX alone provoked lethal effects only at the doses of 90 μg/kg (3/8 mice: 2/8 within 24 h and 1/8 after 8 days from the treatment) and 270 μg/kg (2/8 mice within 24 h). OA administration (370 μg/kg) did not result in lethal effects (Table 8).

No symptoms or signs of toxicity were recorded in mice administered with the lowest dose of PLTX (30 μg/kg) in association with OA (370 μg/kg). On the contrary, after co-administration of the mid and the highest doses of PLTX (90 and 270 μg/kg) and OA (370 μg/kg), almost all the mice showed scratching, as recurrent episodes starting from 30 min post dose and recovering within few hours. Prior to death, the signs of

toxicity observed in mice administered with the mid and highest doses of PLTX in association with OA were similar to those observed after the administration of the corresponding doses of PLTX alone, but were amplified by the co-presence of OA. They included piloerection, abdominal dilation accompanied by hunched posture and ataxia, followed by spasms, paralysis, mainly of the hind limbs, accompanied by sternal or lateral recumbency and respiratory distress (mainly dispnoea). No alterations were recorded in animals given OA alone and no severe signs of diarrhea were noted throughout the observation period (Table 9).

Table 8: Incidence of mortality after acute oral administration of PLTX and OA.

Group (No. of animals)	Doses of PLTX (µg/kg)	Doses of OA (µg/kg)	Study day (number of animals)
1 (16F)	0	0	(0)
2 (16F)	0	370	d1(1)
3 (8F)	30	0	(0)
4 (3F)	90	0	d1(2); d9(1)
5 (8F)	270	0	d1(2)
6 (8F)	30	370	(0)
7 (8F)	90	370	d1(2)
8 (8F)	270	370	d4(1); d5(1)

Worsening of the general clinical conditions accompanied by loss in body weight was the general finding recorded in all animals prior to unscheduled sacrifice death. Mortality did not show a clear dose-relationship or single toxin effect, however, the highest mortality was noted in the higher toxin combination group, PLTX 270 µg/kg + OA 370 µg/kg.

After single administration of the control or OA 370 µg/kg, no changes in the detailed behavior and/or clinical signs were observed. In the PLTX 30 µg/kg group alone and the combined PLTX 30 + OA 370 µg/kg, a high incidence of scratching was observed at approximately 3 hours from treatment. In particular, all animals treated with PLTX

alone and animals treated with PLTX and OA in combination. Severity was higher in the groups treated with PLTX alone.

Table 9: Clinical signs following single oral administration of PLTX and OA.

	Control	OA 370 µg/kg	PLTX 30 µg/kg	PLTX 90 µg/kg	PLTX 270 µg/kg	PLTX 30µg/kg OA 370µg/kg	PLTX 90µg/kg OA 370µg/kg	PLTX 270µg/kg OA 370µg/kg
Scratching	0	0	0	+	0	++	0	0
Jumping	0	0	0	0	+	0	0	0
Paralysis	0	0	0	0	++	0	++	++
Tremor activity	0	0	0	0	+	0	0	+
Abnormal gait	0	0	0	++	+	0	+	++
Respiratory rate	0	0	0	0	+	0	+	0
Diarrhoea	0	++	++	0	0	0	0	0
Righting reflex	0	0	0	0	0	0	+	0
Sedation	0	0	0	++	+	0	+	+
Reactivity to touch	0	0	0	+	++	0	+	+++
Piloerection	0	0	0	+	+	+++	+	++
Articular spasms	0	0	0	+	+	0	+	+
Abdominal dilation	0	0	0	+	0	0	0	+
Hypersensitivity to light	0	0	0	0	+	0	0	0

Legend: 0 within normal values; + increase frequency; ++ medium increase in frequency; +++ high increase in frequency; ++++ highly increased frequency

Food Intake and body weight

Minimal to slight reduction in food intake was noted in all treated groups over the entire duration of the study. Inter-individual cage variability was quite high mainly in the second study.

Each animal was weighed before the beginning of treatment and then daily throughout the study period.

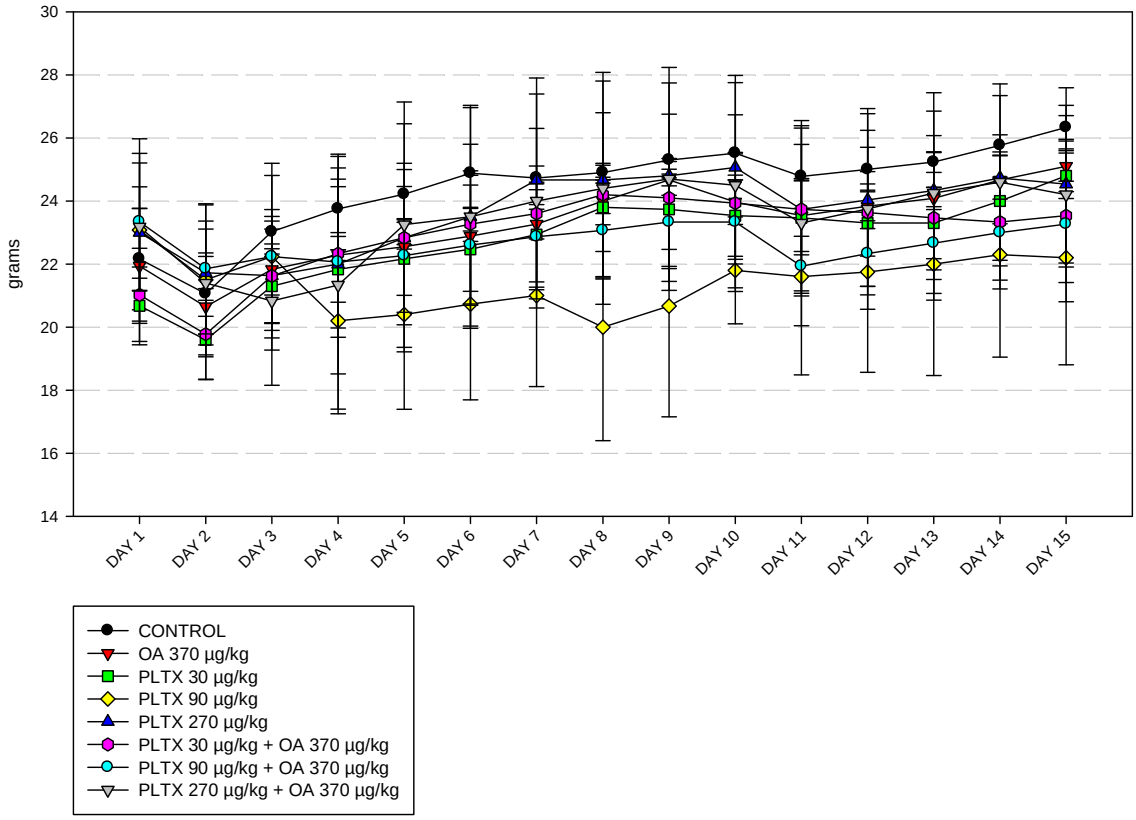
At 24 h after the treatment, no significant differences in body weight between control mice and mice co-administered with PLTX and OA, or with each toxin alone, were recorded. Similarly, no major treatment-related effects on body weight were seen in the subgroups of three mice observed during the recovery period, up to 14 days after the treatment. Only mice administered with PLTX at the dose of 90 μg/kg showed a decreased body weight starting on day 4 up to day 9 (Figure 1).

No major treatment-related effects on body weight and body weight growth were seen in animals of the various groups. Trends were similar. Immediately after administration (day 2), a slight decrease was observed also in the control group. Physiological increase in body weight, as is expected of animals of this age, concurred, but in general at a few grams less than those observed in the control group. Nevertheless, effects on the body weight due to the vehicle cannot be completely ruled out. Recovery of normal weight gain was recorded starting from the third day after treatment. The body weight growth over the study was slightly lower in animals dosed with higher level of PLTX, without an evident dose relationship which can be observed as a minor mean difference at the end of the study between mice of different groups versus control. The lower body weight gain was recorded in mice treated with PLTX 90 μg/kg in the order of

about 5 grams decrease starting from day 4 onwards. The different combinations of toxins did not apparently have effect the growth in body weight.

The single oral dose of 90 µg/kg PLTX led to a higher decrease in body weight in particular starting on day 4. Concurrent clinical signs and biochemistry analysis did not show any noteworthy findings which could reflect the decrease of body weight. Following study day 10 a slight increase in body weight was observed in these animals, but nevertheless remained below the general trend of the animals.

Figure 3 : Body weight following acute oral administration of PLTX and OA (mean ± SD).



Gross pathology and organ weight

At necropsy, 2/8 mice sacrificed at 24 h after the administration of the lower dose of PLTX (30 μg/kg) in association with OA (370 μg/kg) showed pale fluid accumulation in the upper part of the small intestine. On the other hand, a significant number of mice sacrificed at 24 h or spontaneously died within 24 h after the administration of the mid and highest doses of PLTX (90 or 270 μg/kg) in association with OA (370 μg/kg) showed redness at the gastrointestinal wall and/or pale liquid accumulation in the upper part of the small intestine (6/8 and 6/8 mice).

Redness of the intestinal wall, but with lower frequency, was observed also in some mice administered with PLTX alone (2/8 mice at 90 μg/kg: one mouse sacrificed at 24 h and one died after 8 days; 1/8 mice at 270 μg/kg at 24 h), while 2/8 mice administered with OA alone showed pale fluid accumulation in the proximal part of the small intestine.

No macroscopic alterations were noted in the large intestine or in the other internal organs in mice sacrificed 24 h after the treatment or in those spontaneously died. Moreover, no alterations were recorded in mice sacrificed at the end of the withdrawal period.

Organs weight evaluation in mice sacrificed at 24 h after the toxins administration showed a non-dose related decrease in liver weight when compared to the concurrent controls (about 30 %) only in mice administered with the higher dose of PLTX (270 μg/kg) alone or in combination with OA (370 μg/kg). No significant changes in the main organs weight between control and toxins treated mice were recorded after 14 days of recovery (Figures 4, 5 and 6).

Figure 4: **Absolute organ weights in mice at 24 h after acute oral administration of palytoxin and okadaic acid (mean ± SD).**

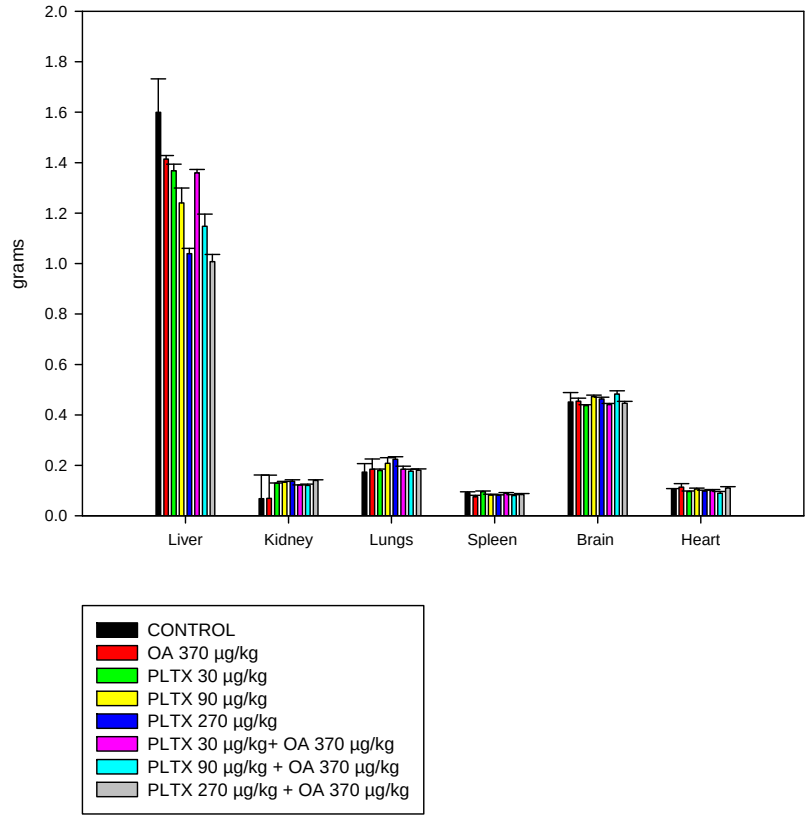


Figure 5: **Absolute organ weights in mice 14 days after acute oral administration of palytoxin and okadaic acid (mean ± SD).**

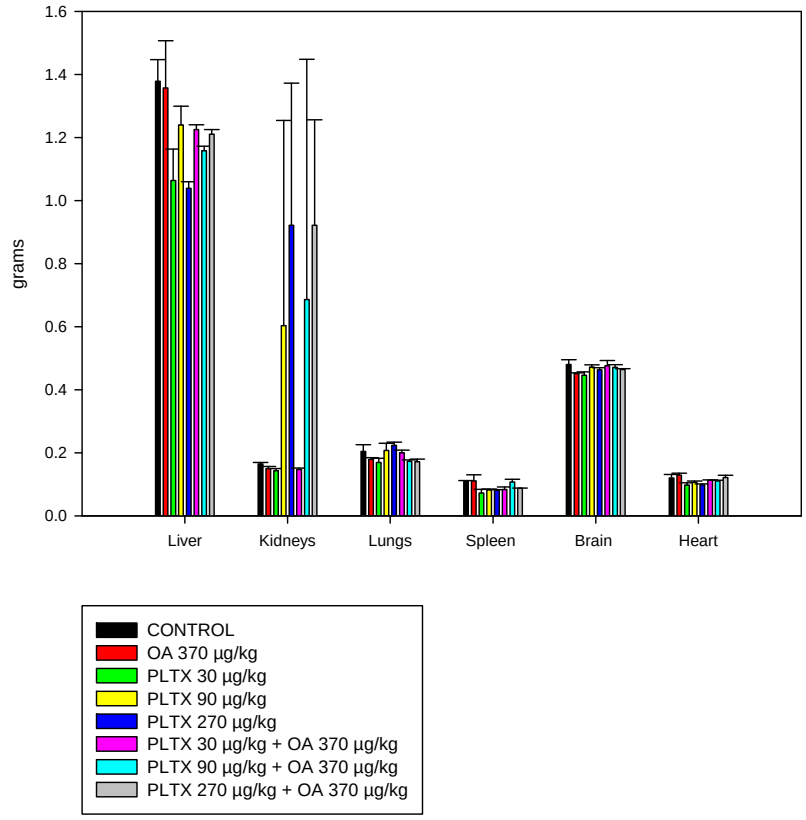
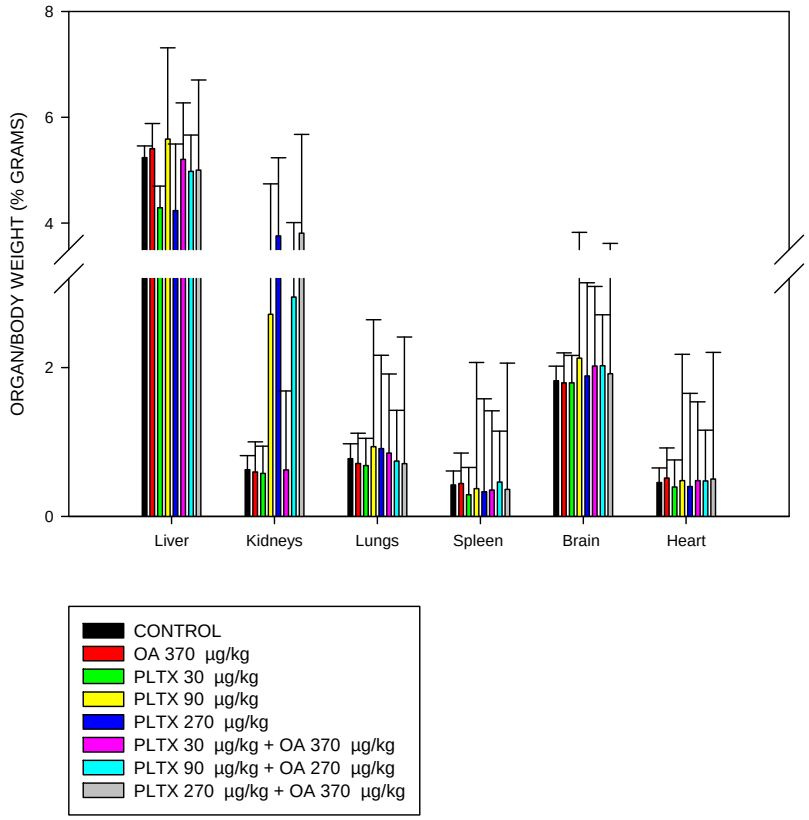


Figure 6: **Relative organ/body weights 14 days following acute oral administration of PLTX and OA (mean ± SD).**



Clinical Chemistry

No evident treatment-related changes in clinical chemistry parameters were recorded in mice following single oral administration of palytoxin and okadaic acid, in combination or as single toxins. In particular, 24 h after the toxins administration, only mice administered with the lowest dose of PLTX (30 μg/kg) in association with OA (370 μg/kg) showed a reduction in serum levels of alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST) and glutamate dehydrogenase (GLDH), cholesterol (CHL), glucose (GLU), triglycerides (TG), blood urea nitrogen (BUN), total proteins (TP), albumin (ALB) and globulins (GLOB) (Figures 7, 9, 11). In addition, mice administered with the mid dose of PLTX (90 μg/kg) associated to OA (370 μg/kg) showed only a decreased serum glucose level (Figure 9). Hematochemical parameters in the other groups of mice were not significantly different from those of control mice.

After 14 days of recovery, no significant changes in clinical chemistry parameters were recorded in mice administered with PLTX and OA association or with each toxin alone with respect to controls (Figures 8, 10, 12).

No changes were observed at 24 hours following treatement or 14 days of recovery on the ions investigated.

Figure 7: **Serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) in mice 24 h after acute oral administration of palytoxin and okadaic acid.**

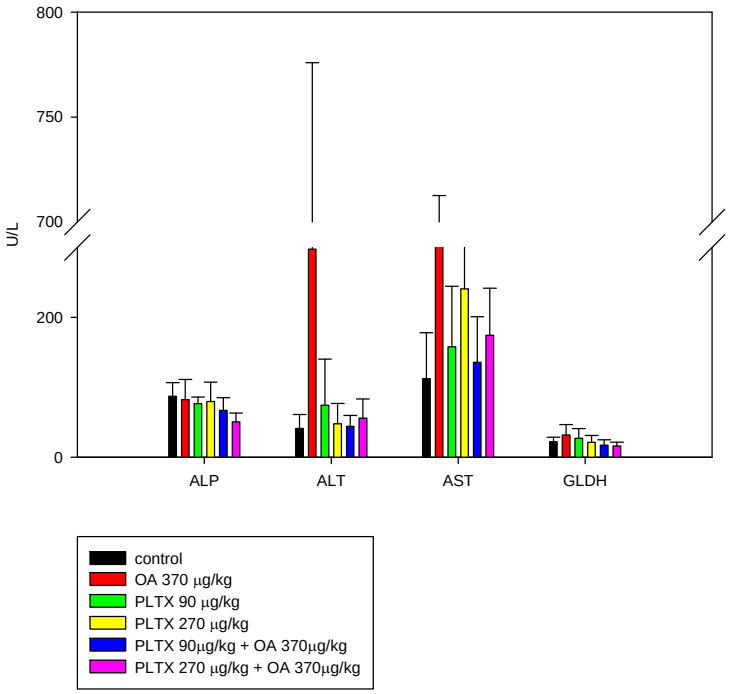


Figure 8: **Serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) in mice 14 days after acute oral administration of palytoxin and okadaic acid.**

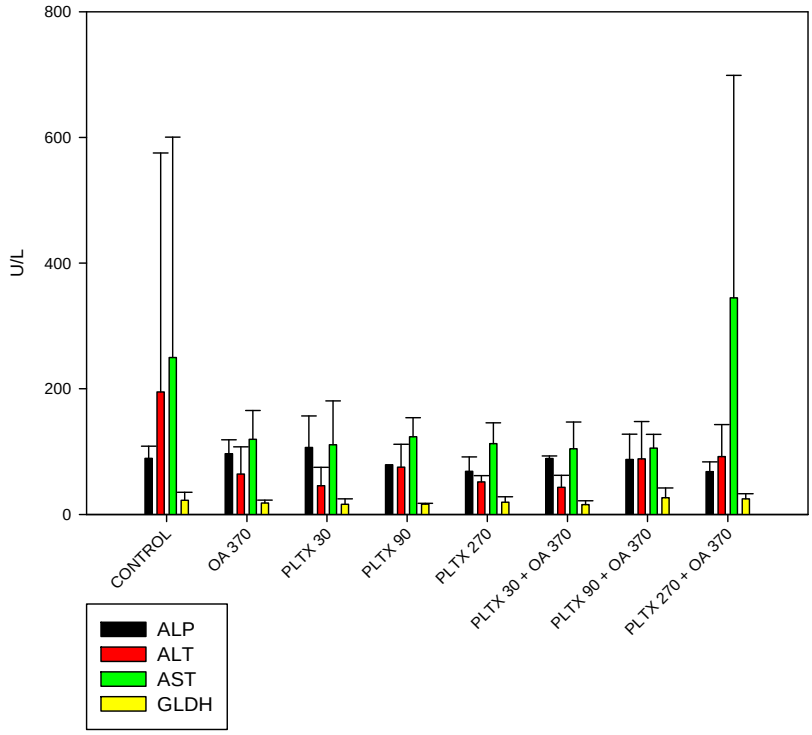


Figure 9: **Serum levels of cholesterol (CHL), triglycerides (TG), glucose (GLU), creatinine (CRE) and blood urea nitrogen (BUN) in mice 24 h after acute oral administration of palytoxin and okadaic acid.**

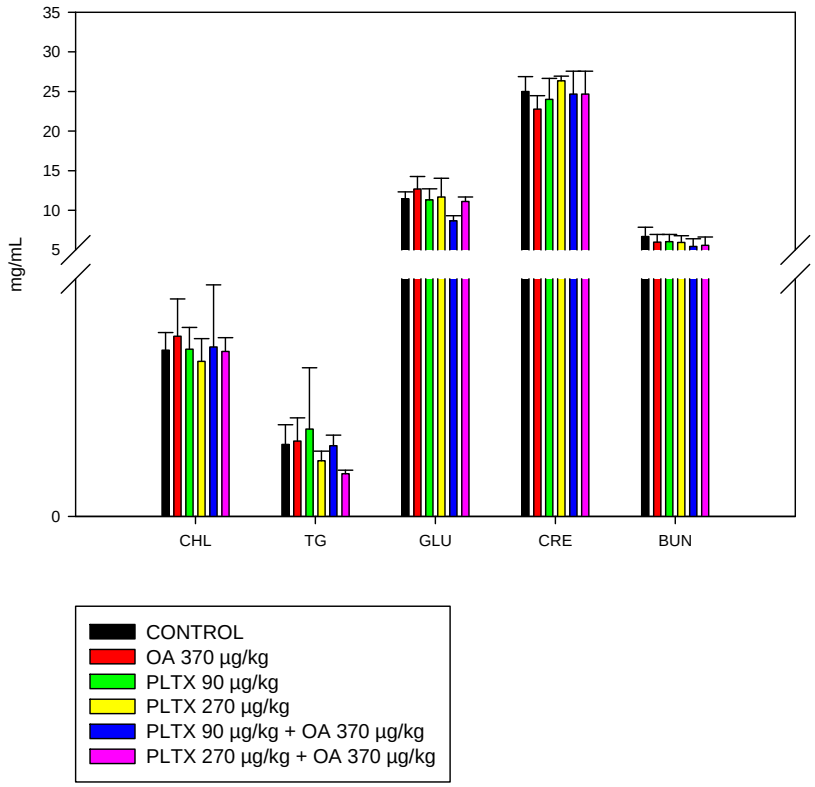


Figure 10: **Serum levels of cholesterol (CHL), triglycerides (TG), glucose (GLU), creatinine (CRE) and blood urea nitrogen (BUN) in mice on day 14 after acute oral administration of palytoxin and okadaic acid.**

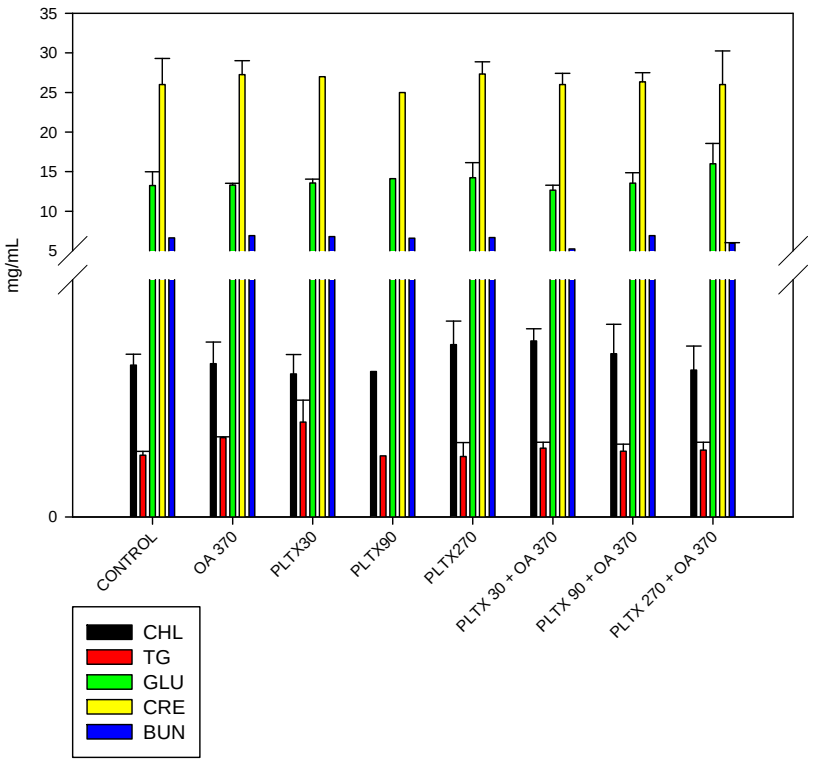


Figure 11: **Serum levels of total proteins (TP), albumin (ALB) and globulins (GLOB) in mice 24 h after acute oral administration of palytoxin and okadaic acid.**

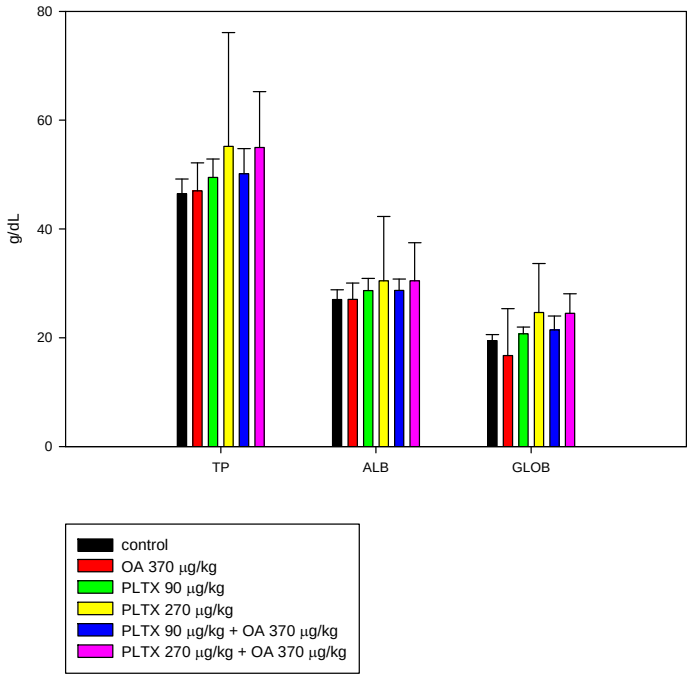


Figure 12: **Serum levels of total proteins (TP), albumin (ALB) and globulins (GLOB) in mice 14 days after acute oral administration of palytoxin and okadaic acid.**

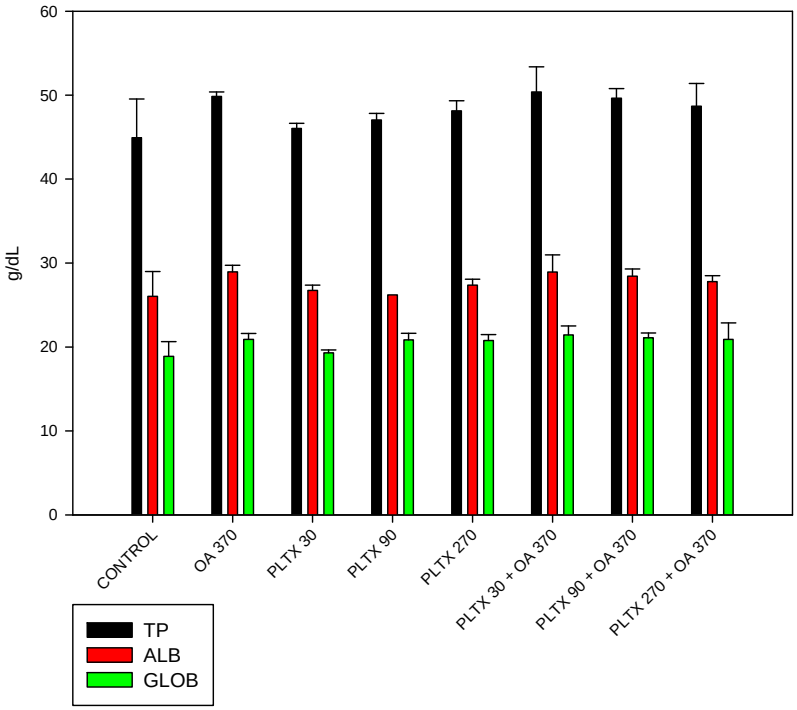


Figure 13: **Serum levels of Ca^{2+}, inorganic phosphorous (Pi), Na^{+}, K^{+} and Cl^{-} in mice 24 h after acute oral administration of palytoxin and okadaic acid.**

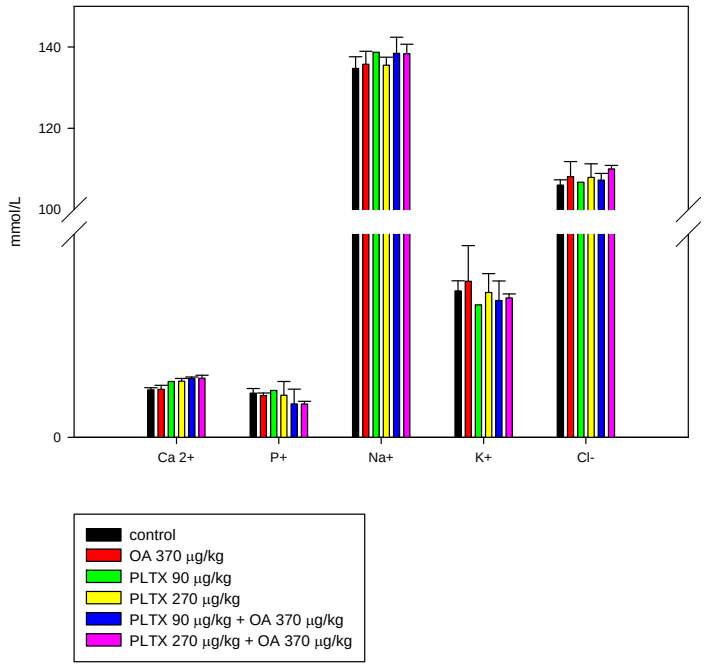
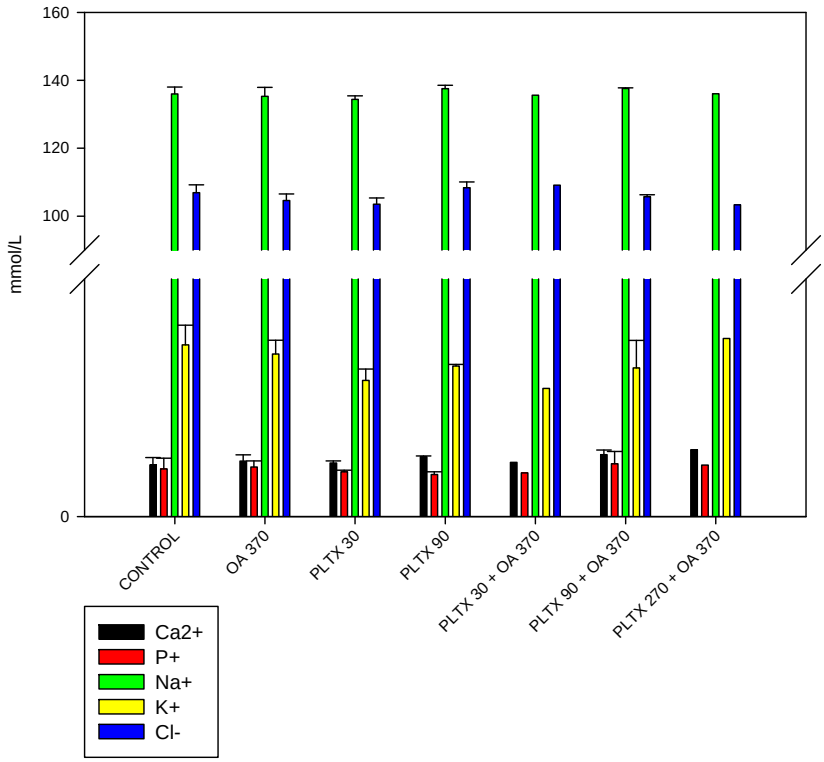


Figure 14: **Serum levels of Ca^{2+}, inorganic phosphorous (Pi), Na^{+}, K^{+} and Cl^{-} in mice 14 days after acute oral administration of palytoxin and okadaic acid.**



Light microscopy histological pathology

Histology revealed that oral co-administration of PLTX and OA caused changes in gastric and hepatic tissues. Tissue changes were noted in animals sacrificed 24 h after administration; no alterations were recorded in mice after the withdrawal period of 14 days.

At the hepatic level, a decrease of glycogen content in hepatocytes was observed in almost all animals given the highest PLTX dose (270 µg/kg), alone or in combination with OA (370 µg/kg) (Figure 15).

At the gastric level, slight to mild ulcers accompanied by inflammatory infiltrations of the fore-stomach was observed in 2/5 animals dosed with 90 µg PLTX/kg associated with 370 µg OA/kg and in 2/5 mice administered with 270 µg PLTX/kg associated to 370 µg OA/kg. Slight inflammatory infiltrates typical of gastritis, including hyperaemia, oedema and polymorphonuclear cells infiltrate was observed in the forestomach of 2/5 animals administered with the highest dose of PLTX (270 µg/kg) combined with OA (370 µg/kg) (Figures 16 and 17).

Figure 15: **Micrographs of the liver. Decrease in hepatic glycogen content was recorded at 24 h after the administration in all PLTX treated animals starting from the dose of 90 µg/kg with an unclear dose relationship. Magnificatio 10x. Control animal on top; PLTX 270 µg/kg + OA 370 µg/kg on bottom.**

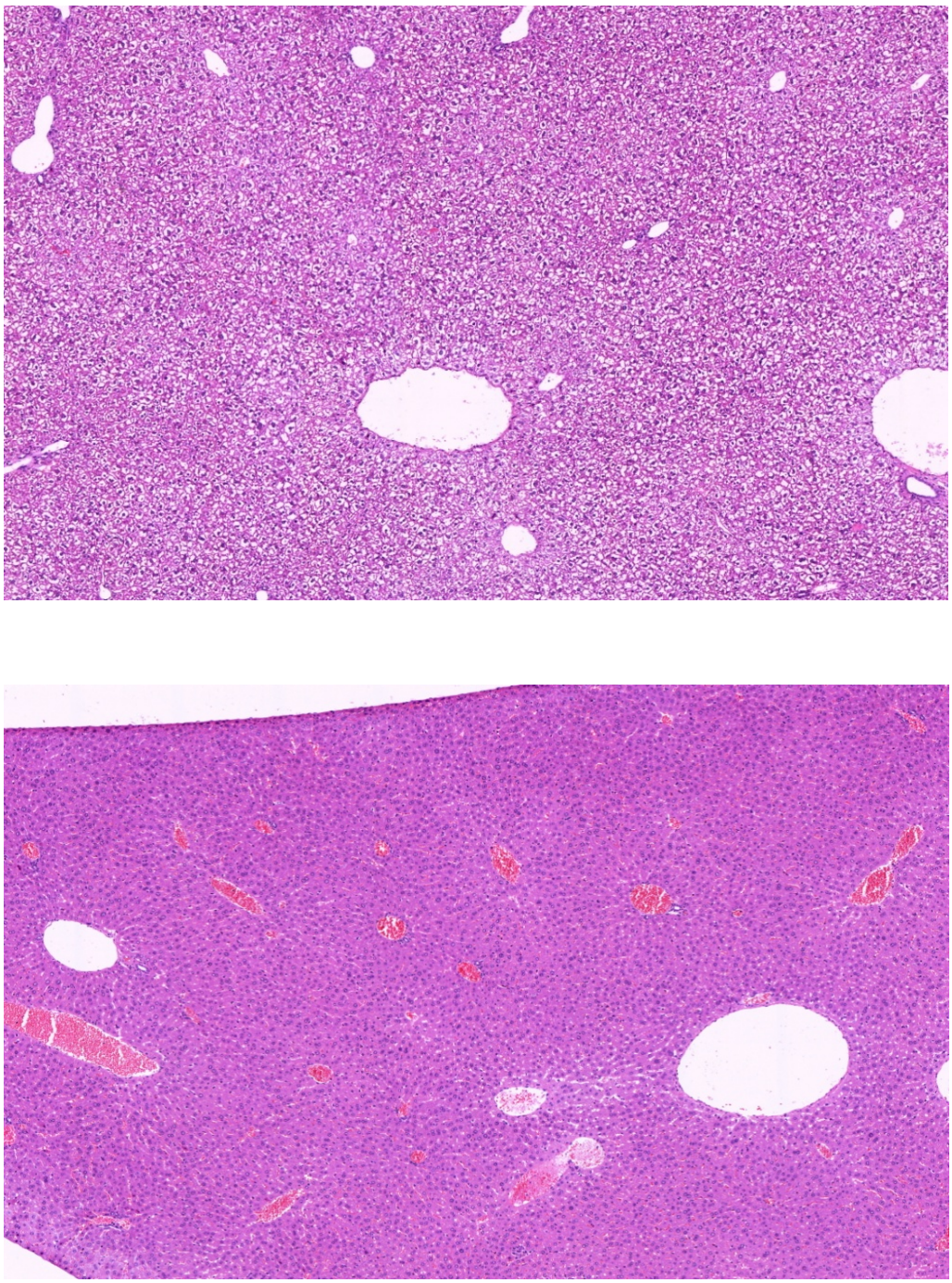


Figure 16: **Micrographs of the stomach. Slight to marked acute inflammation of the submucosae in the non glandular stomach associated with a slight focal ulcers in some animals treated with PLTX 90 µg/kg and OA 370 µg/kg. Complete recovery was observed at the end of the withdrawal period. Magnification 10 x. Control animal on top, treated on bottom.**

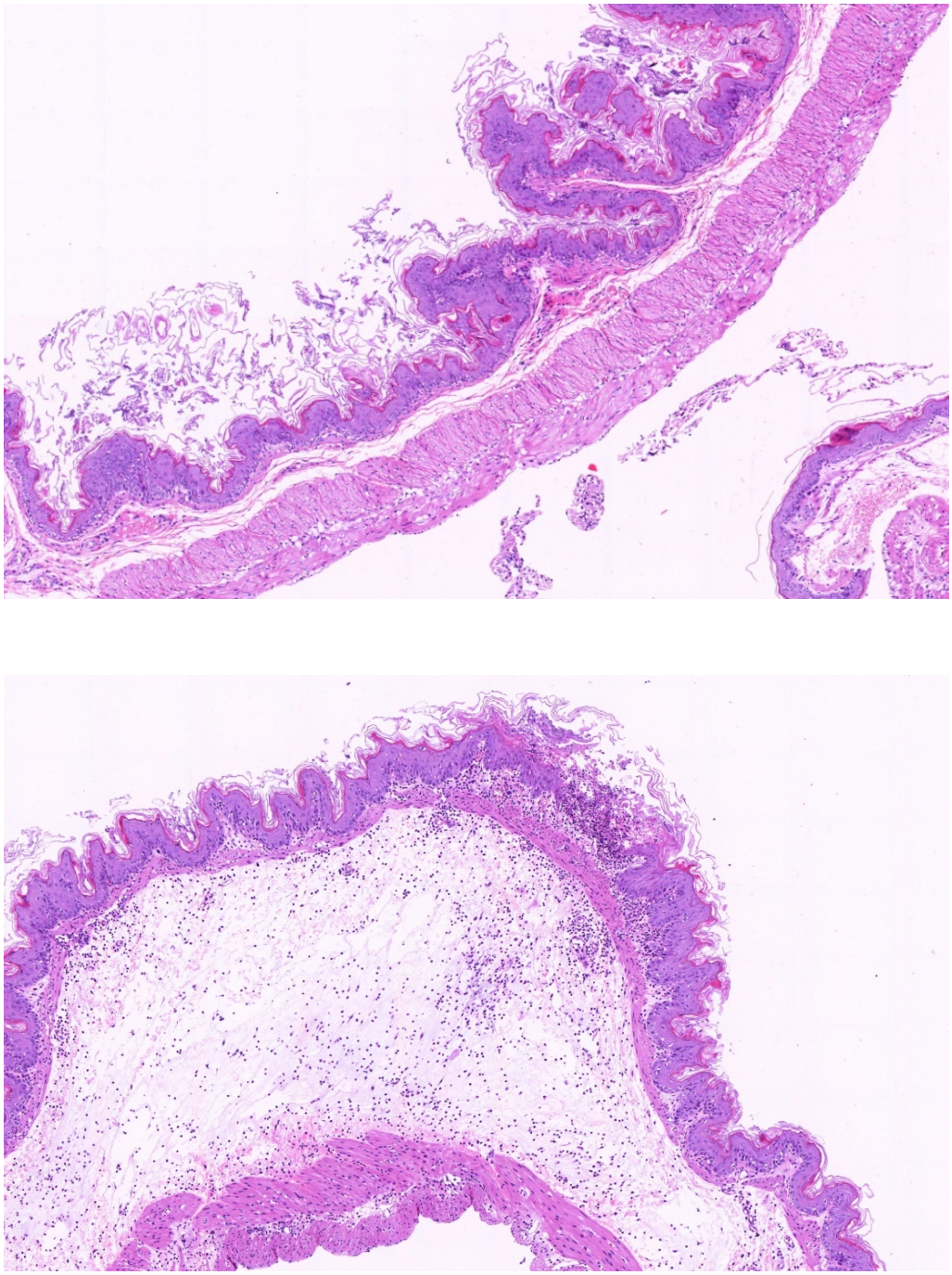
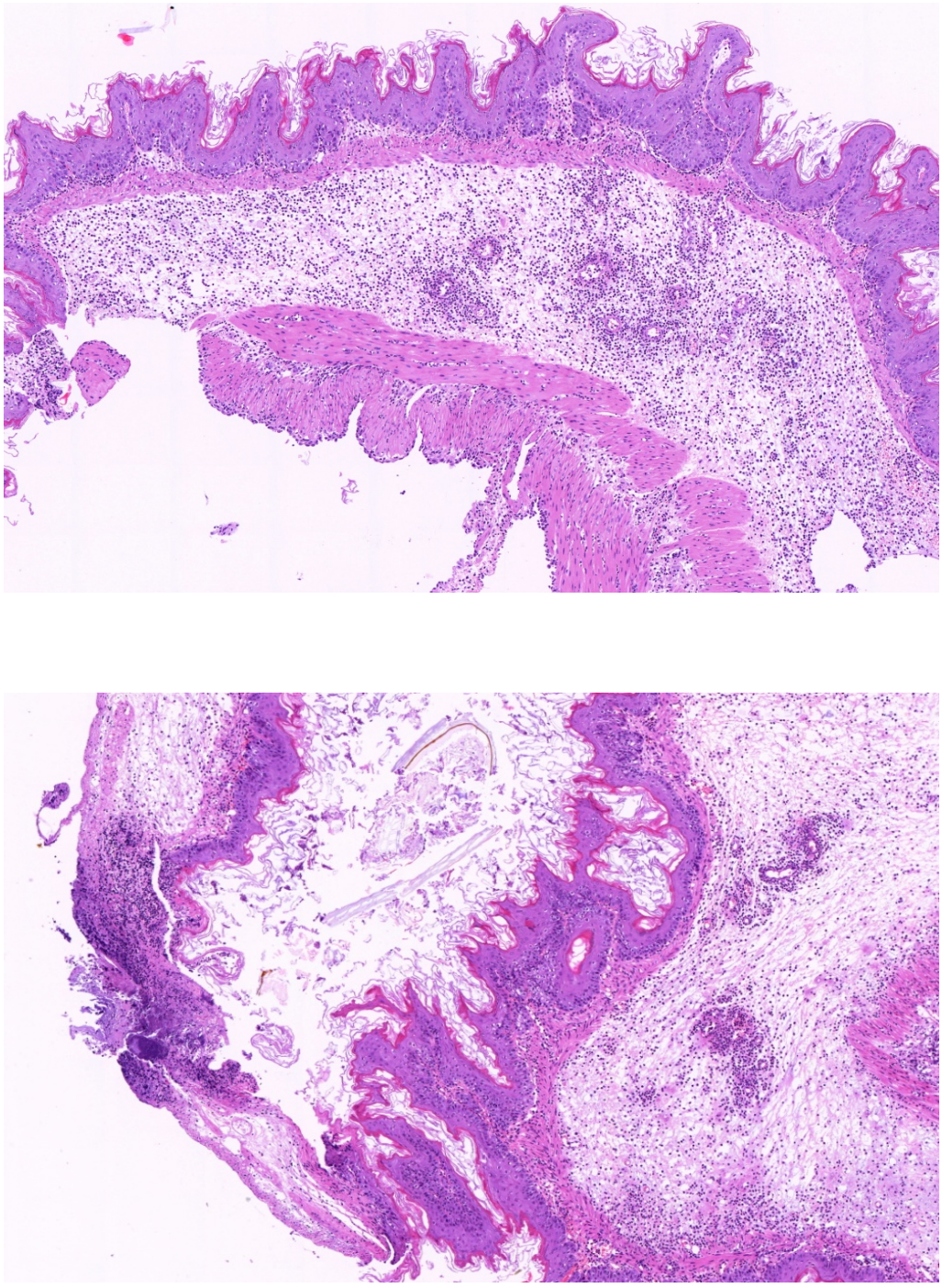


Figure 17: **Micrographs of the stomach. Slight to marked acute inflammation of the submucosae in the non glandular stomach in some animals treated with PLTX 270 µg/kg + OA 370 µg/kg. Complete recovery was observed at the end of the withdrawal period. Magnification 10X.**



TOXICITY OF PALYTOXIN AFTER REPEATED ORAL ADMINISTRATION

The toxic effects due to the daily repeated oral administration of palytoxin in mice were investigated as a preliminary study before the evaluation of combined repeated oral doses of palytoxin and okadaic acid effects. To this aim, mice were daily administered, for 7 days, with oral doses of palytoxin and the toxic effects were evaluated up to 24 h after the last treatment (the first scheduled time of sacrifice, for subgroups of 3 or 5 mice) and up to 14 days after the last treatment (the second scheduled time of sacrifice, for subgroups of 3 mice).

To this aim, two set of experiments have been carried out: since all the PLTX doses used in *Experiment 1* (30, 90, 180 μg/kg/day, administered to groups of 6 mice) induced some toxic effects, *Experiment 2* was designed in order to identify a no effect level and the doses of 3 and 30 μg PLTX/kg/day were administered to groups of 8 mice.

Mortality and Clinical signs

During *Experiment 1*, the repeated oral administration of palytoxin caused mortality prior to scheduled sacrifices at all the doses, i.e. 30, 90 and 180 μg/kg/day. In particular, PLTX caused lethal effects in 2/6 mice at the dose of 30 μg/kg/day (at 6^{th} and 8^{th} days of treatment: d6 and d8), 2/6 mice at the dose of 90 μg/kg/day (d3 and d7) and 5/6 mice at the highest dose (3 at d3, 1 at d5 and 1 at d6) (Table 10). During the recovery period, 1/6 mice treated with the dose of 30 μg/kg/day died three days after the last treatment (d10), while at the dose of 90 μg/kg/day 2/6 mice died three and five days after the last treatment (d10 and d12). The only mouse administered with the highest toxin dose surving the treatment also survived during the recovery period (Table 10).

No mortality was observed during *Experiment 2*, after the daily administration of 3 and 30 µg PLTX/kg/day (Table 10).

Table 10: Lethality of mice during the daily oral administration of palytoxin and the recovery period.

Dose of PLTX (µg/kg/die)	Day of death (number of animals)
First experiment	
Control	-
30	d4 (1/6) d8 (1/6) d10 (1/6)
90	d3 (1/6) d7 (1/6) d10 (1/6) d12 (1/6)
180	d3 (3/6) d5 (1/6) d6 (1/6)
Second experiment	
Control	(0/8)
3	(0/8)
30	(0/8)

During *Experiment 1*, mice died during the treatment period showed typical signs and symptoms. The most common signs observed prior to death were scratching, breathing difficulties, abnormal gait, lethargy, and paralysis of the hind limbs. All clinical signs observed were in a clearly dose dependant manner starting from the dose of 30 µg/kg/day. Independently from lethal effects, abdominal swelling was visible starting from d3 in 4/6, 3/6 and 1/6 animals administered with 30, 90 and 180 µg/kg/day, respectively. Furthermore, chromodacryorrhoea was observed in 2/6 and 1/6 mice treated with 30 and 90 µg PLTX/kg/day, starting from d3 and d4, respectively. No particular

clinical signs were observed in animals surviving on day 21 (all control animals, 2/6 animals dosed at 30 µg/kg; 1/6 at 90 µg/kg; 1/6 at 180 µg/kg). Clinical signs reported in Table 11 refer to the 21 days of observation and to the number of surviving animals of each group.

Table 11: Clinical signs in mice after repeated oral administration of palytoxin.

	Control	PLTX 30 µg/kg	PLTX 90 µg/kg	PLTX 180 µg/kg
Scratching	0	++	+	++
Abdominal dilatation	0	+++	++	+++
Breath difficulty	0	+	++	+++
Paralysis	0	+	+	++

Legend: 0 within normal values; + increase frequency; ++ medium increase in frequency; +++ high increase in frequency; ++++ highly increased frequency

In *Experiment 2*, mice treated with 3 µg PLTX/kg/day showed no alterations or clinical signs in comparison to controls. Mice administered with 30 µg PLTX/kg/day showed slight transient hypoactivity and sporadic scratching episodes. In addition, abdominal swelling was recorded in 2/8 mice, starting from d5 or d7. No changes were observed in gait or posture.

Food intake and body weight

During *Experiment 1*, a reduced daily food intake was recorded in mice administered with all the toxin doses, starting from d2. It reached 60 % (30 µg/kg/day), 47 % (90 µg/kg/day) and 49 % (180 µg/kg/day). Inter-individual cage variability was quite high. During the recovery period, food consumption by surviving mice slowly returned comparable to that of control mice (Figure 18).

Also during *Experiment 2*, a reduced food consumprion (24-48 %) was recorded in mice administered with 30 µg/kg/day of palytoxin, from d4 to d10. Subsequently, it became compoarable to that of controls. On the contrary, mice administered with the lowest dose of palytoxin (3 µg/kg/day) did not show differences in food consumption with respect to control mice during the whole period of observation (Figure 19).

Figure 18: **Food consumption of mice recorded during palytoxin administration and the recovery period in *Experiment 1*.**

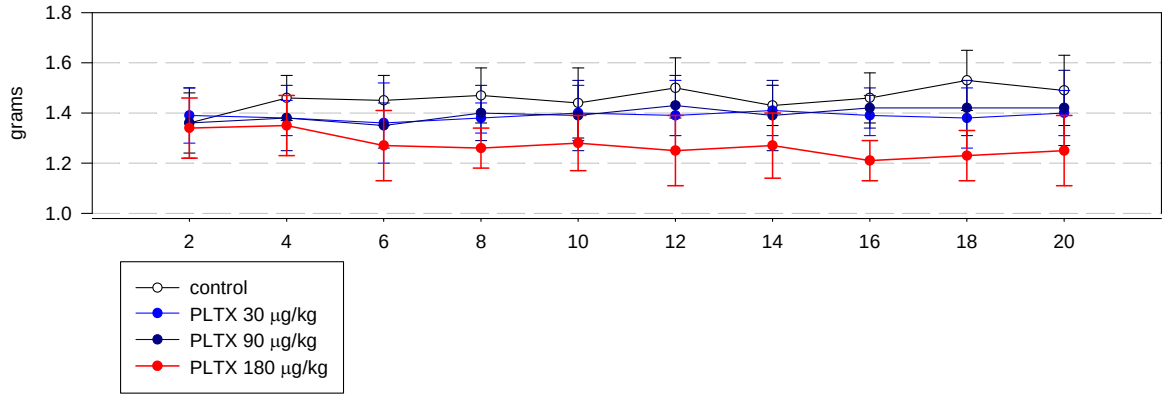
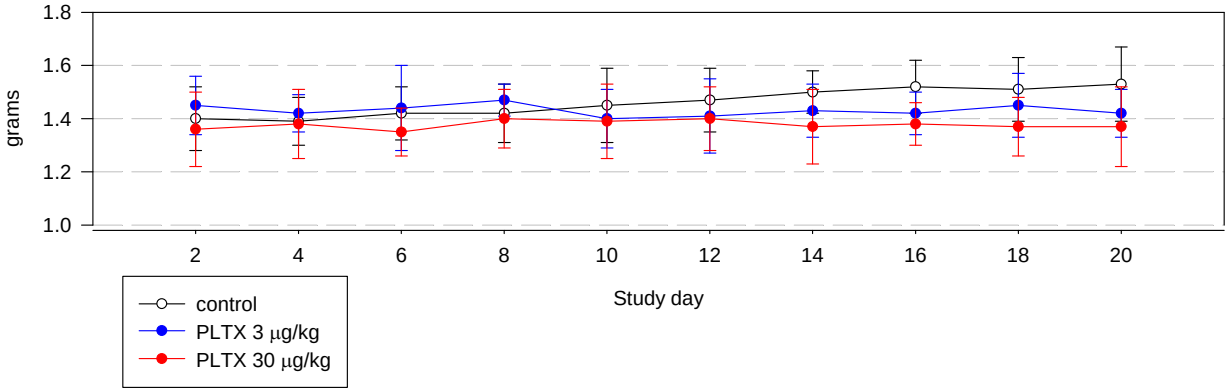


Figure 19: **Food consumption of mice recorded during palytoxin administration and the recovery period in *Experiment 2*.**



Considering the body weight, in *Experiment 1* a reduction of body weight of mice (up to 40 %) was recorded in all the groups administered with palytoxin as compared to controls. In particular, although a slight body weight decrease due to gavage was observed also in the control group at day 2, the body weight gain in mice treated with palytoxin was in general at a few grams lower than that of controls. In control mice, a recovery of normal weight gain was recorded starting from the third day after treatment. The body weight growth over the entire period of observation was lower in animals dosed with higher level of PLTX, without an evident dose relationship.. The lowest body weight gain was recorded in mice treated with PLTX at 180 µg/kg/day in the order of about 5 grams decrease starting from day 4 onwards (Figure 20).

In *Experiment 2*, a reduced body weight (up to 25 %) was recorded at the dose of 30 µg PLTX/kg/day starting from d4 throughout d8, whereas no changes in body weight were observed in mice administered with the lowest toxin dose (3 µg/kg/day) (Figure 21).

Figure 20: ***Experiment 1*** **- Body weight of mice recorded during palytoxin administration (day 1–day 7) and the recovery period (day 8–day 21). The first scheduled sacrifice was performed 24 h after the end of treatment period.**

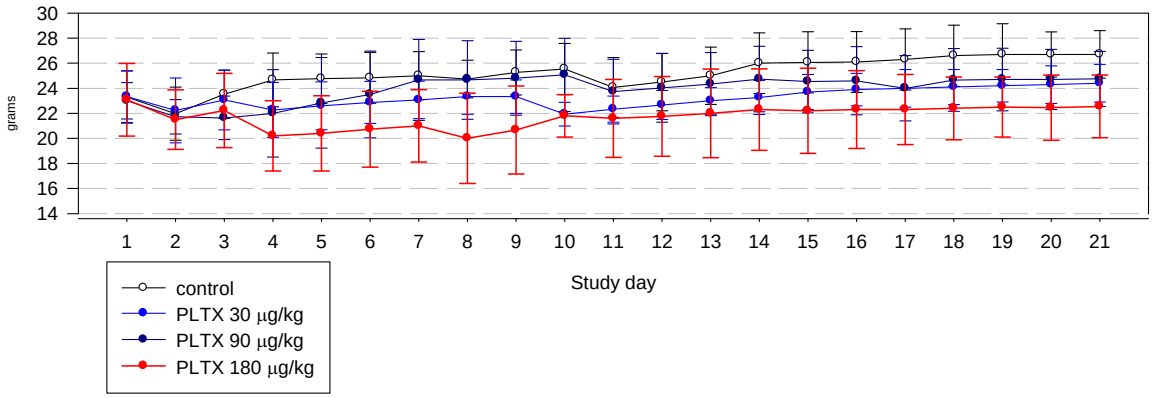
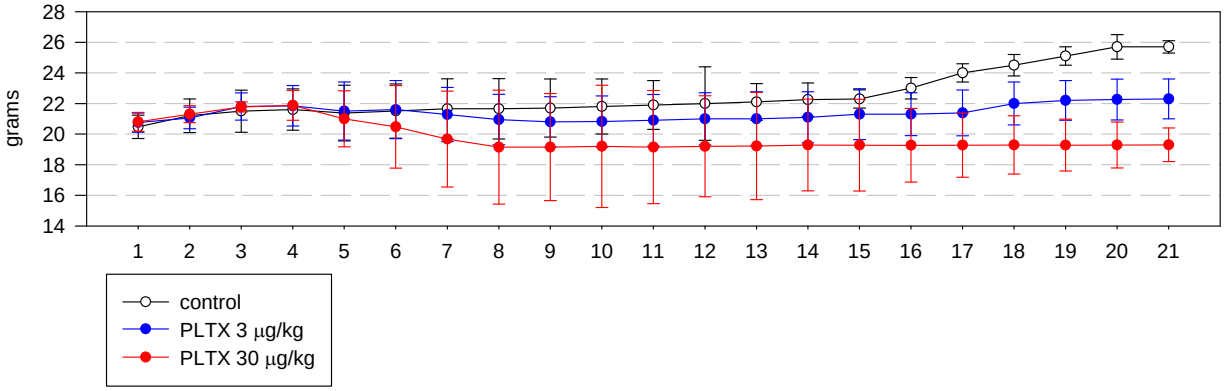


Figure 21: ***Experiment 2*** **- Body weight of mice recorded during palytoxin administration (day 1–day 7) and the recovery period (day 8–day 21). The first scheduled sacrifice was performed 24 h after the end of treatment period.**



Gross pathology and organ weight

In *Experiment 1*, necropsy of PLTX treated mice showed abdominal swelling corresponding to massive liquid and gas accumulation in the intestinal tract, associated to lower fecal content than in control mice. Furthermore, in 1/6 mice treated with 180 µg PLTX/kg/day dead during the treatment period, focal areas of discoloration were observed in the liver. Within 24 h after the last treatment, increases in the relative organs weight, expressed as organs/body weight ratios, were also observed, reaching statistical significance only at the highest dose of PLTX (180 µg/kg/day): spleen (53% increase), heart (43%) and kidneys (27%). In addition, a non significant increase of lungs/body weight ratio (55–98%) was recorded at all PLTX doses (Figure 24). At the end of the recovery period, the relative organs weights of mice treated with PLTX were similar to those of control mice (Figure 24). The same trend could be observed for relative organ to body weight ratios.

During *Experiment 2*, no changes in relative organs weights were recorded at 3 and 30 µg PLTX/kg/day, at the end of the treatment or at the end of the recovery period (Figure 27).

Figure 22: ***Experiment 1*** **- Absolute organ weights in mice on day 8 after repeated oral administration of palytoxin (mean ± SD); n=5.**

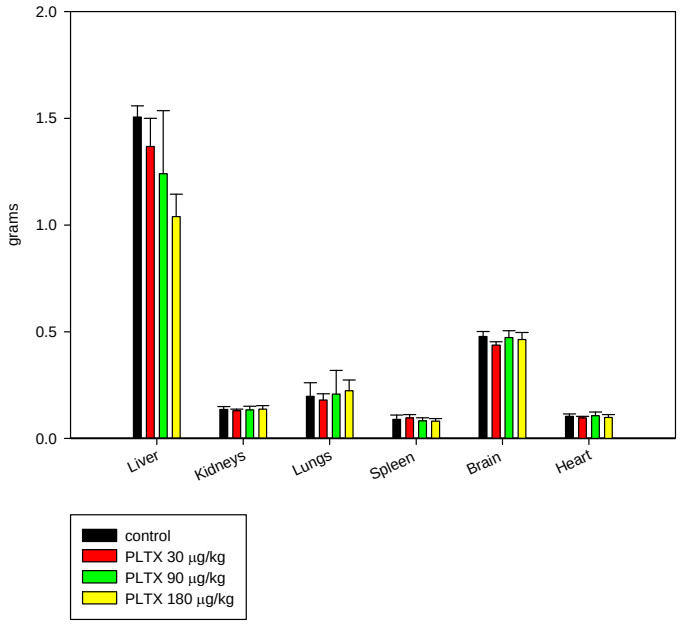


Figure 23: ***Experiment 1*** **- Absolute organ weights in mice on day 21 after repeated oral administration of palytoxin (mean ± SD); n=3**

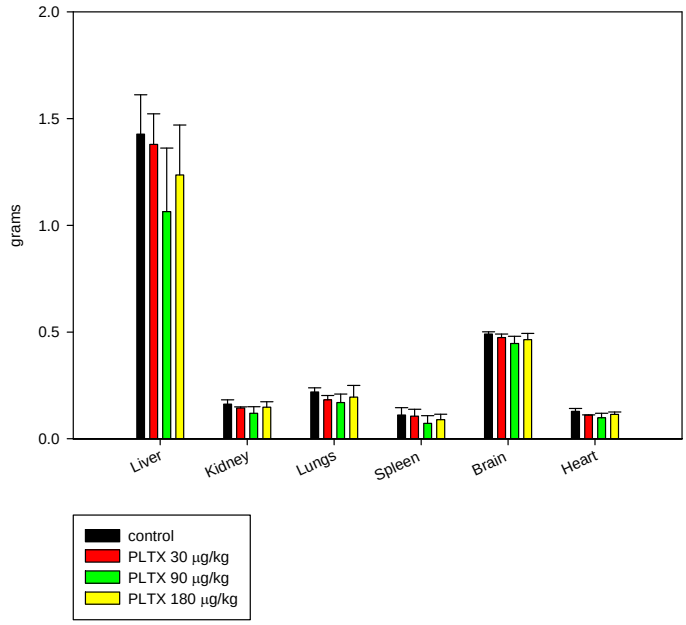
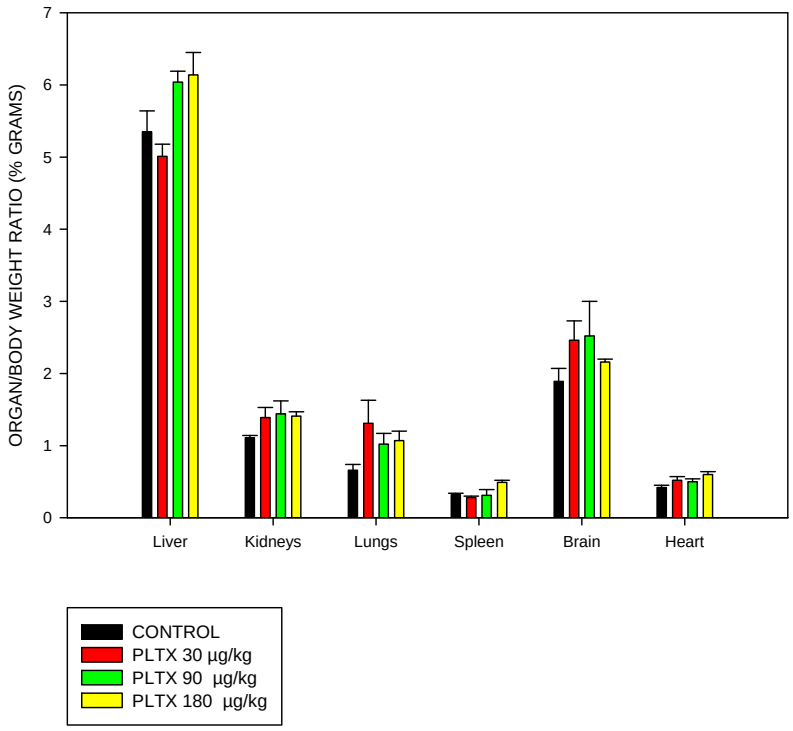


Figure 24: ***Experiment 1*** **- Relative organ/body weights on day 8 (top graph) and 14 days (bottom graph) following repeated administration of PLTX (mean ± SD).**



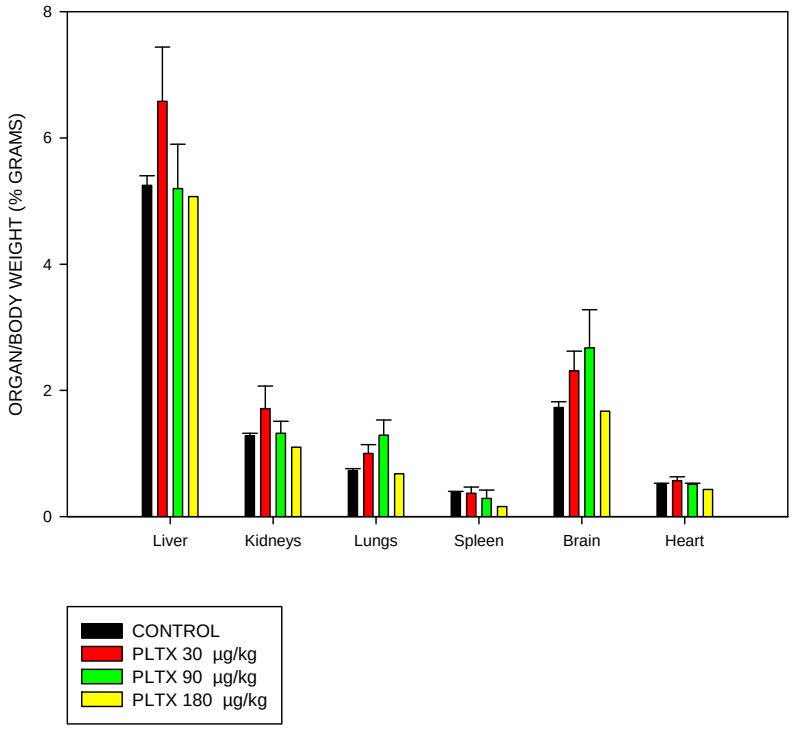


Figure 25: ***Experiment 2*** **- Absolute organ weights in mice on day 8 following repeated oral administration of palytoxin (mean ± SD); n=5.**

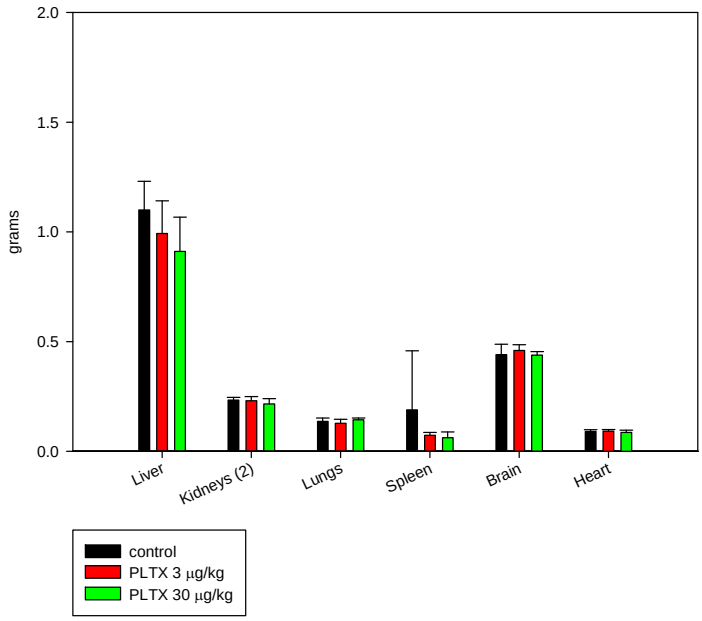


Figure 26: ***Experiment 2*** **- Absolute organ weights in mice on day 21 following repeated oral administration of palytoxin (mean ± SD); n=3.**

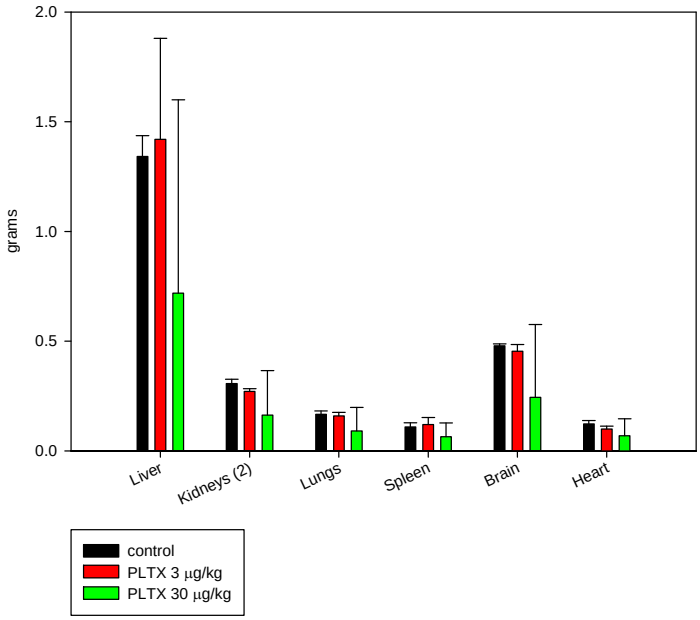
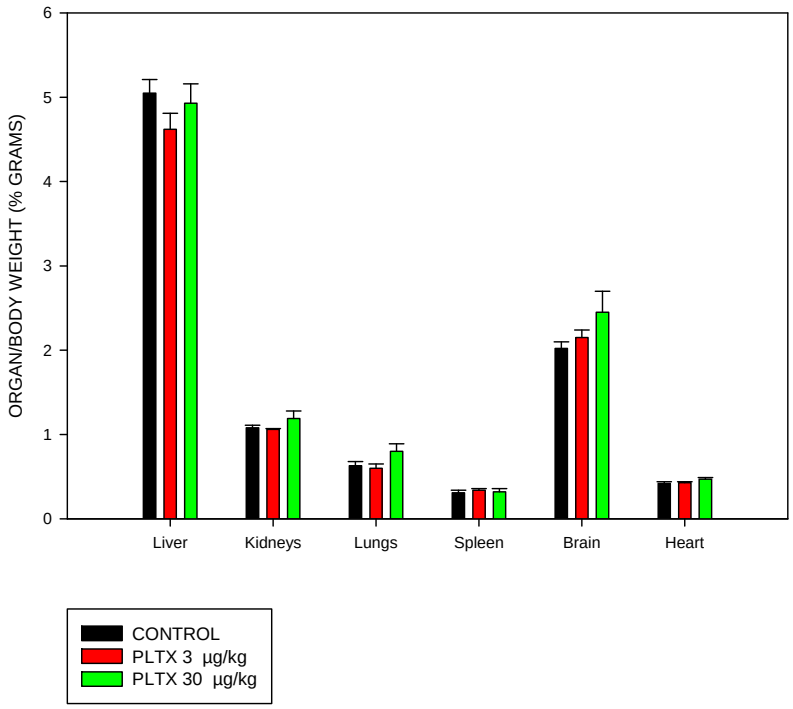
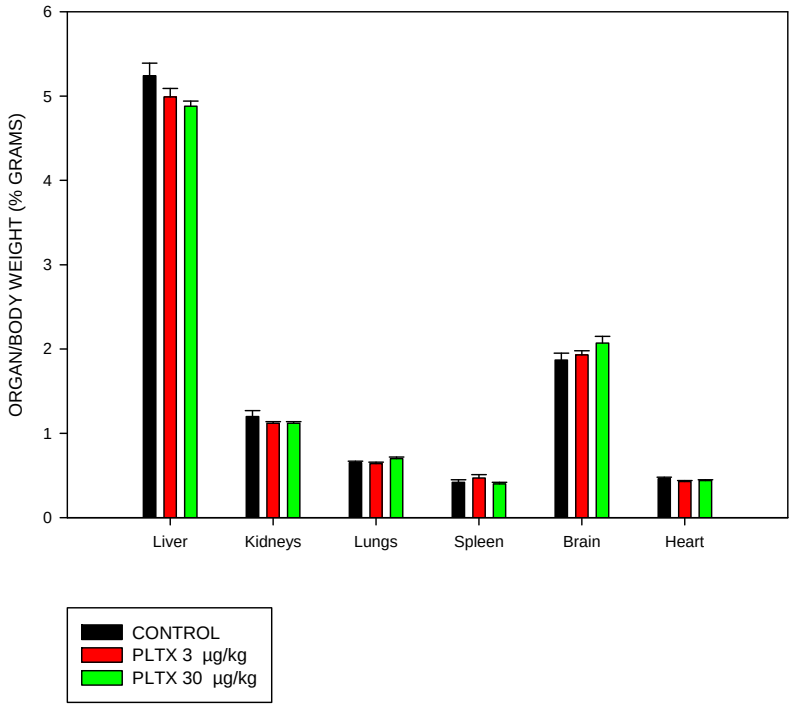


Figure 27: ***Experiment 2*** **- Relative organ/body weights on day 8 (top graph; n=5) and day 14 (bottom graph; n=3) following repeated oral administration of palytoxin (mean ± SD).**





Clinical Chemistry

First experiment- Experiment 1

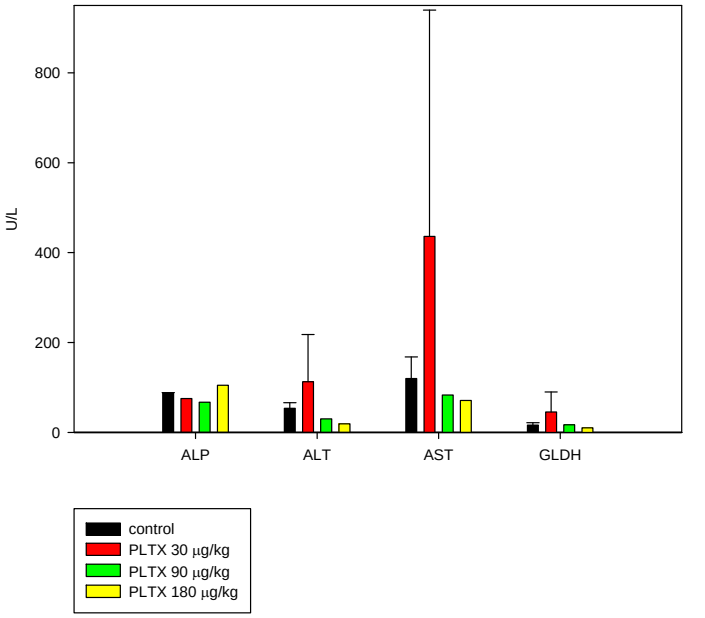
In those animals which were found dead, serum samples could not be obtained.

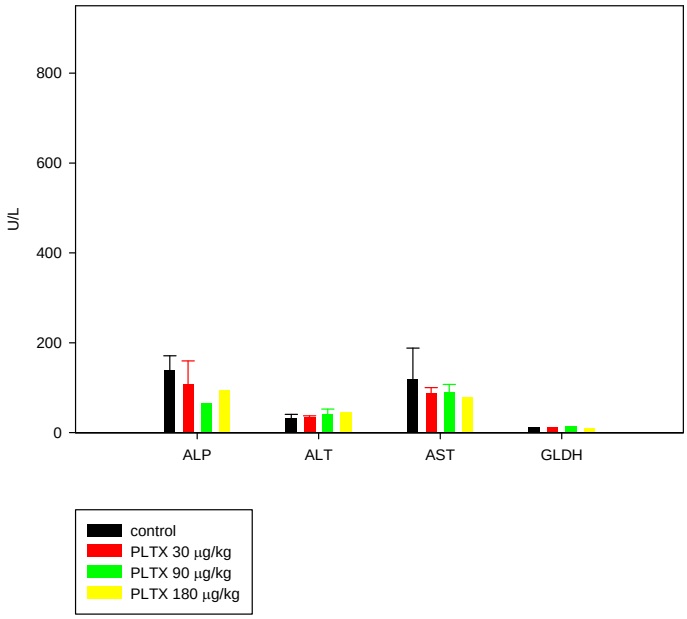
Data presented come from a limited number of animals per group (see mortality). Not all parameters which were chosen for evaluation could be analyzed due to the limited amount which could be sampled from each animal; on day 8 sacrifice values were available only from control animals and animals treated at the highest dose. At the recovery sacrifices from control and 30 μg/kg only.

Graphs show animals sacrificed 24 hours after the last treatment, i.e. day 8 and the remaining surviving animals on day 21.

An increase in all parameters was observed at the lowest treated dose. The finding was not dose dependant. On day 21 an overall decrease in all hepatic enzymes was observed in treated animals again in a non dose dependant manner.

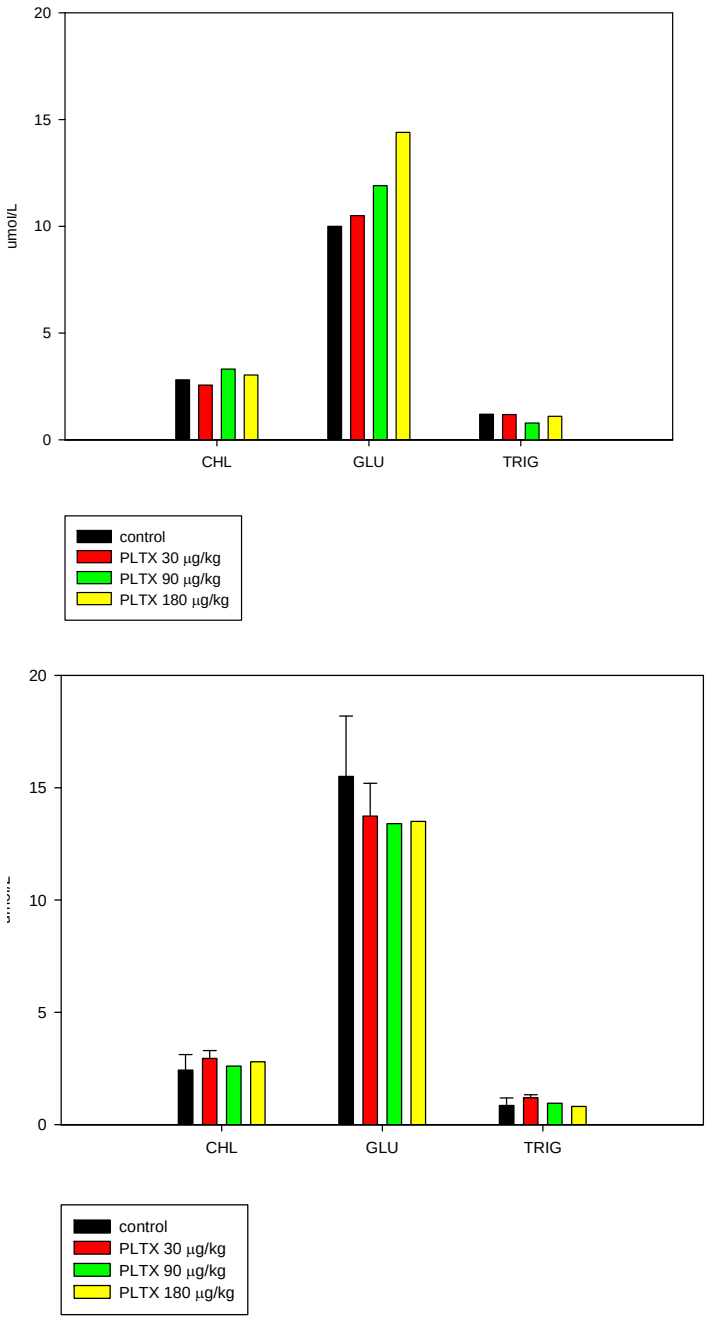
Figure 28: **Serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) in mice at 24 h after daily repeated oral administration of palytoxin (day 8; top graph) or after the recovery period (day 21; bottom graph).**





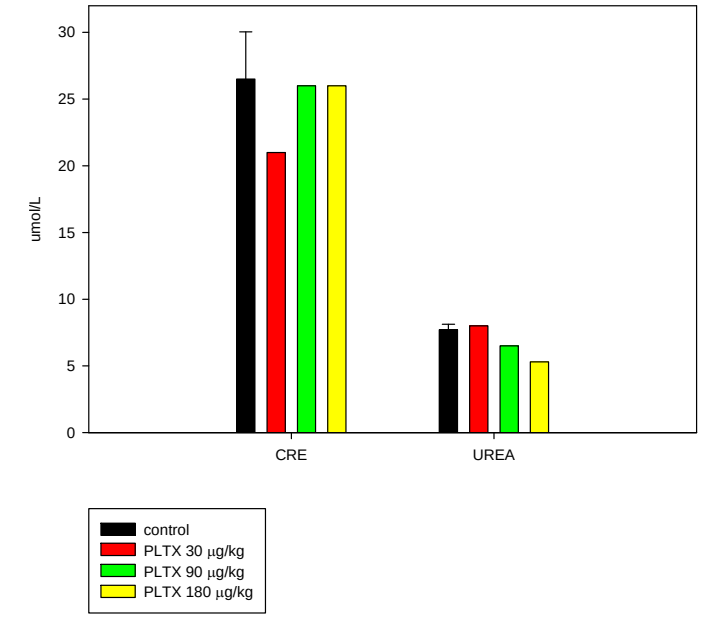
A dose-dependent increase in glucose serum level was observed at d8 , but not at the end of the withdrawal period (d21). No effects on triglycerides or cholesterol were observed (Figure 29).

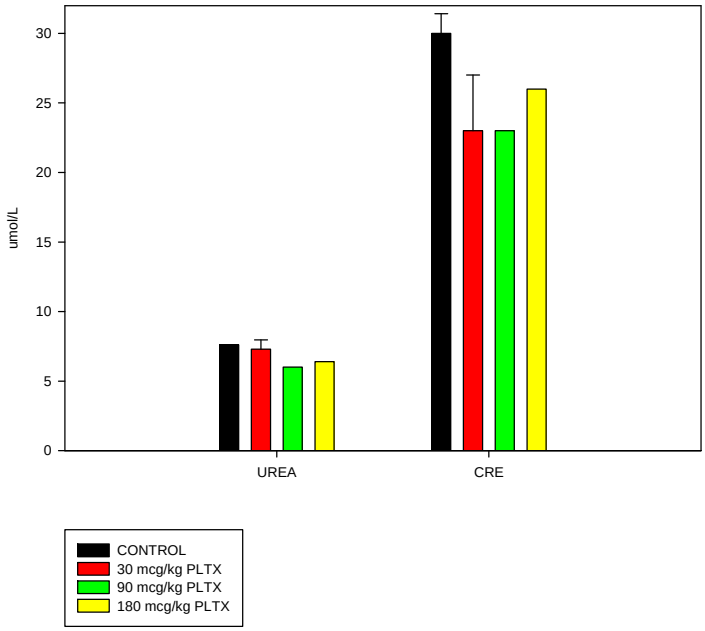
Figure 29: **Serum levels of cholesterol (CHL), glucose (GLU) and triglycerides (TRIG) in mice at 24 h after daily repeated oral administration of palytoxin (day 8; top graph) or after the recovery period (day 21; bottom graph).**



Inconsistent variations in urea and creatinine were observed.

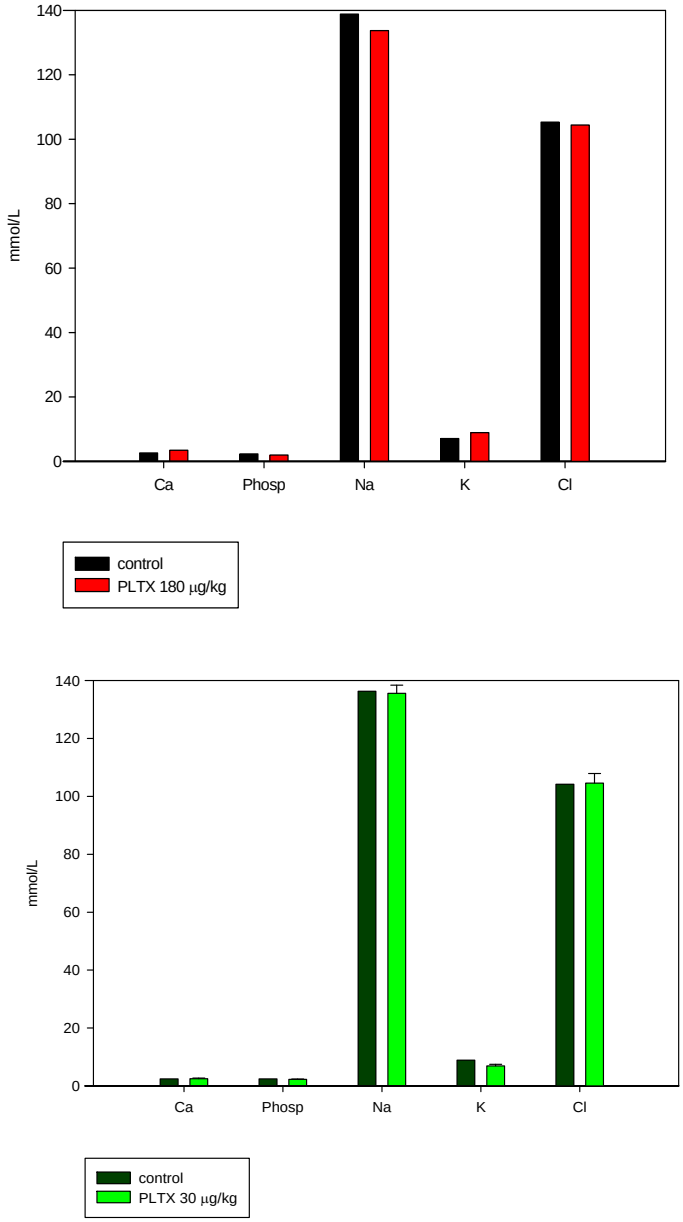
Figure 30: **Serum levels of urea and creatinine (CRE) and urea in mice at 24 h after daily repeated oral administration of palytoxin (day 8; top graph) or after the recovery period (day 21; bottom graph).**





No clear trend in ions variations which could be caused by the repeated oral administration of the compound was observed.

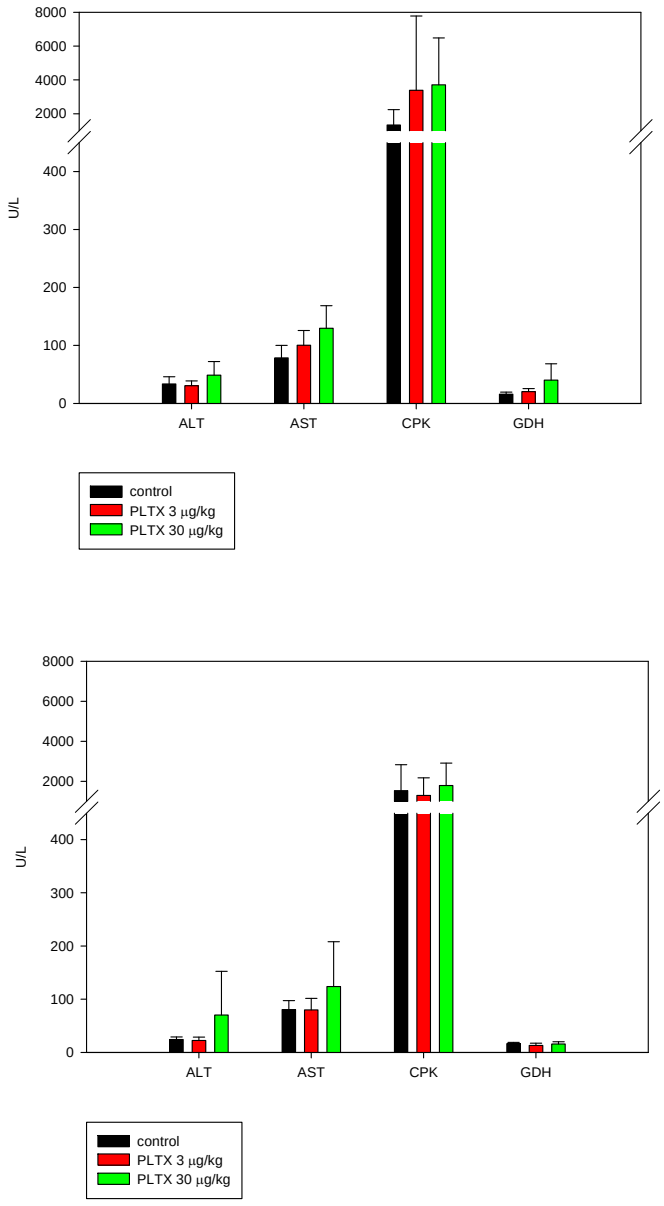
Figure 31: **Serum levels of Calcium (Ca^{2+}), inorganic phosphate (Phosp), Na^+, K^+ and Cl^- in mice at 24 h after daily repeated oral administration of palytoxin (day 8; top graph) or after the recovery period (day 21; bottom graph).**



Second experiment - Experiment 2

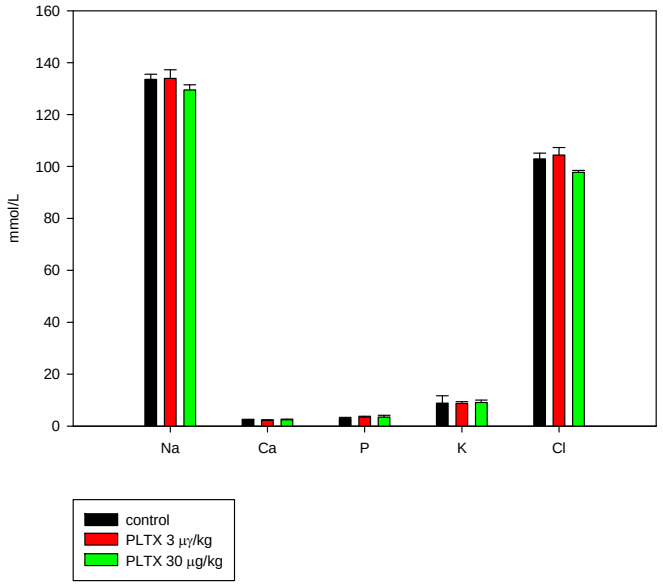
During Experiment 2, a dose-dependent but no statistically significant increase in all assayed parameters was observed on day 8. At the end of the recovery period (d21), values were comparable to those registered in control animals, with the exception of slightly higher, not significant, increase in alanine aminotransferase and aspartate aminotransferase (193% and 54%, respectively), recorded in mice administrered with 30 µg PLTX/kg/day (Figure 32).

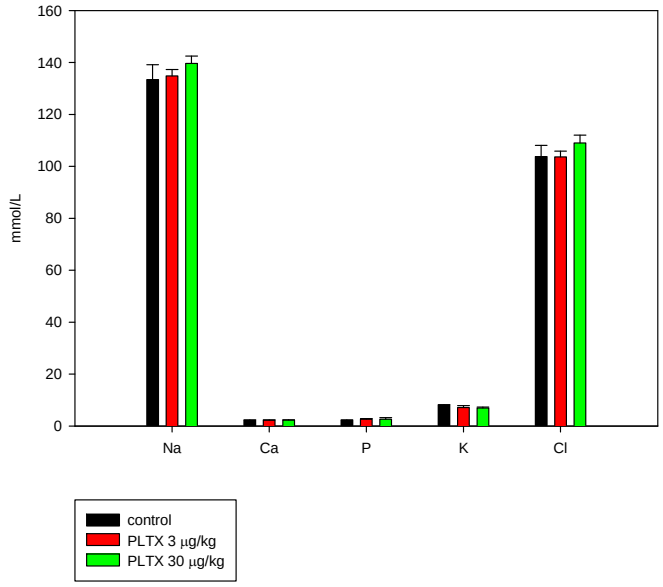
Figure 32: **Serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) in mice at 24 h after daily repeated oral administration of palytoxin (day 8; top graph) or after the recovery period (day 21; bottom graph).**



Clear increase in CPK on day 8 was not associated to an increase in serum potassium level. Minor alterations were noticed in sodium and chloride mainly in animals given the high dose of 30 µg/kg in both scheduled sacrifice.

Figure 33: **Serum levels of Na^+, Ca^{2+}, inorganic phosphate (P), K^+ and Cl^- in mice at 24 h after daily repeated oral administration of palytoxin (day 8; top graph) or after the recovery period (day 21; bottom graph).**





Light microscopy histological pathology

In *Experiment 1*, although no specific cause of death was found in dead mice, multi-organ alterations were observed microscopically throughout the dosed groups which are most likely linked to treatment with palytoxin.

In the lungs, alveolar edema slight to severe in degree, associated in some cases to acute inflammation and necrosis, was observed in mice died prematurely at all doses of palytoxin: 2/6 animals treated with 30 µg PLTX/kg/day, 2/6 treated with 90 µg/kg/day and 2/6 treated with 180 µg/kg/day. No alterations were recorded in control animals (Figure 34)

In one animal treated with 30 µg PLTX/kg/day dead during the treatment priod, the presence of mild gastric ulcers, even if they were limited to the non-glandular stomach, were observed (Figures 35 and 36).

In the liver of all mice died during the treatment period or in those sacrificed at d8, depletion of the glycogen content in the hepatocytes, visible as more a consistent structure of the hepatic cells, was observed. Various degrees of glycogen depletion were noted among the doses groups without relationship with the administered doses. In the liver of 1/6 animal treated with 180 µg/kg of PLTX focal foci of hepatic necrosis were also observed (Figures 37 and 38).

In the spleen of the animals treated with two lower doses of palytoxin (30 and 90 µg/kg/day), a moderate depletion of the lymphoid elements of the lymphatic follicles was observed. The finding was not consistent and not dose dependant since it was not observed at the highest dose.

Figure 34: **Light micrographs of lung from mice of *Experiment 1*. PLTX 180 µg/kg. Alveolar edema, acute inflammation. Magnification 10 x; hematoxylin–eosin stain.**

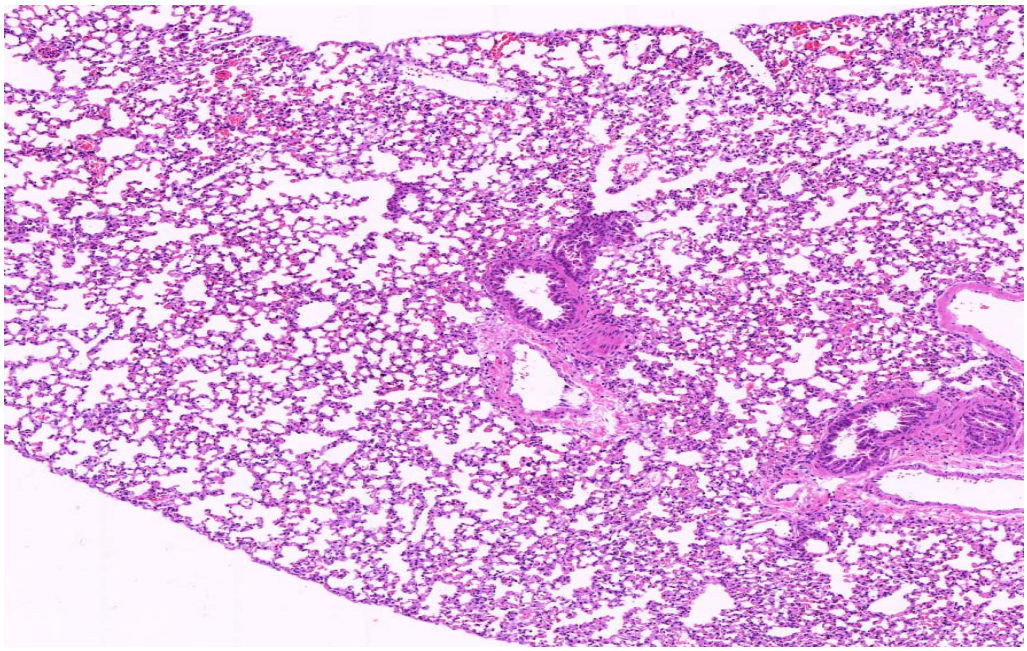


Figure 35: **Light micrographs of non-glandular stomach from mice of *Experiment 1*. Control mouse. Magnification: 10 x; hematoxylin-eosin stain.**

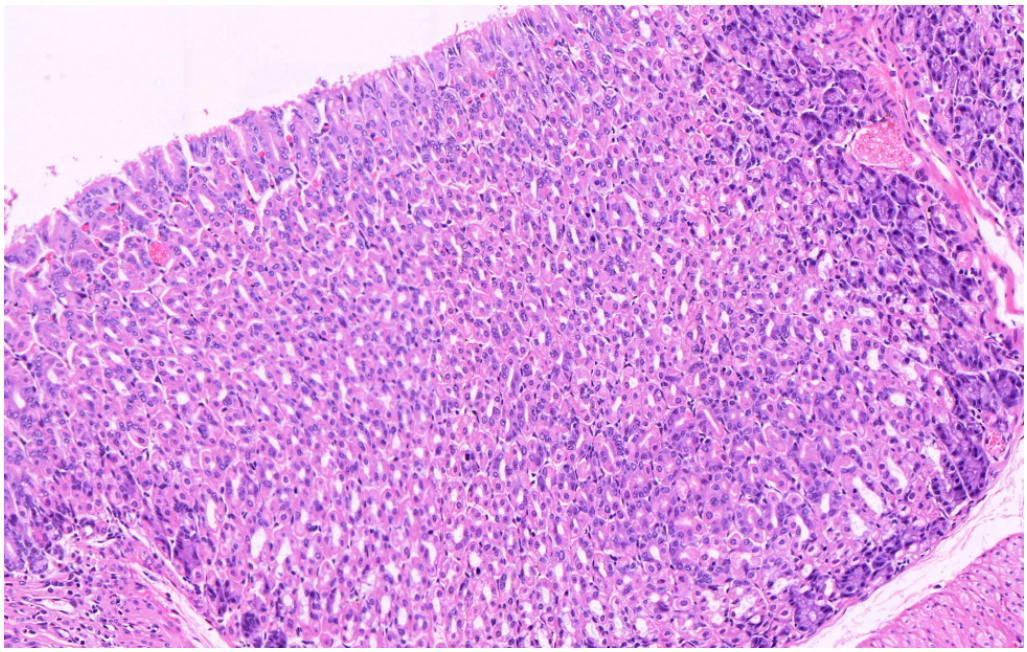


Figure 36: **Light micrographs of non-glandular stomach from mice of *Experiment 1*. PLTX treated mouse at 30 µg/kg/day. Magnification: 10 x; hematoxylin-eosin stain.**

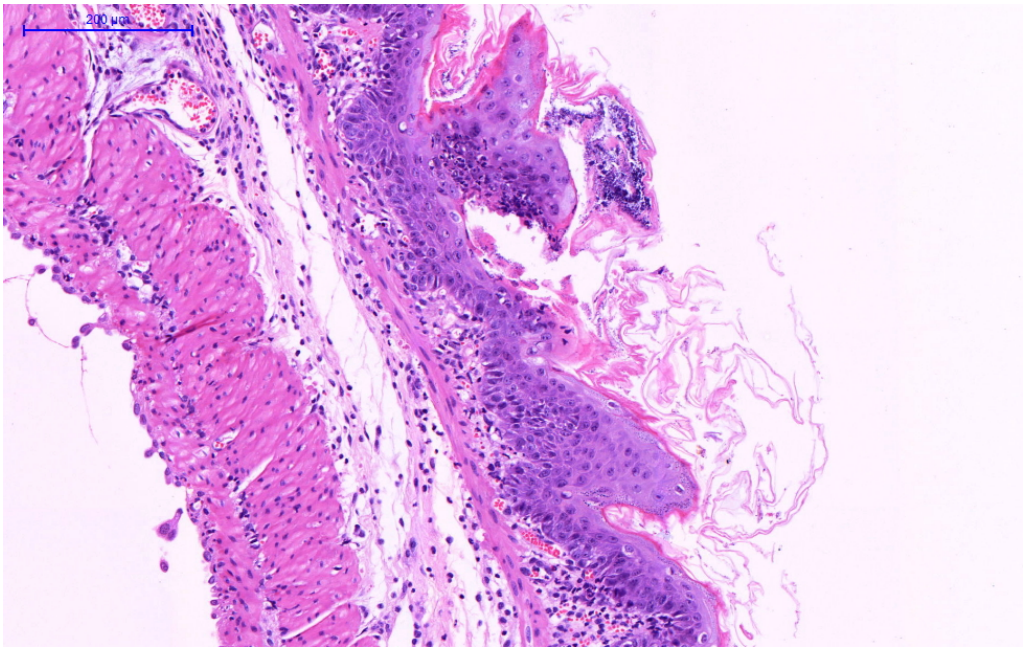


Figure 37: **Light micrographs of liver from mice of *Experiment 1*. Control mouse; Magnification: 10 x; hematoxylin–eosin stain.**

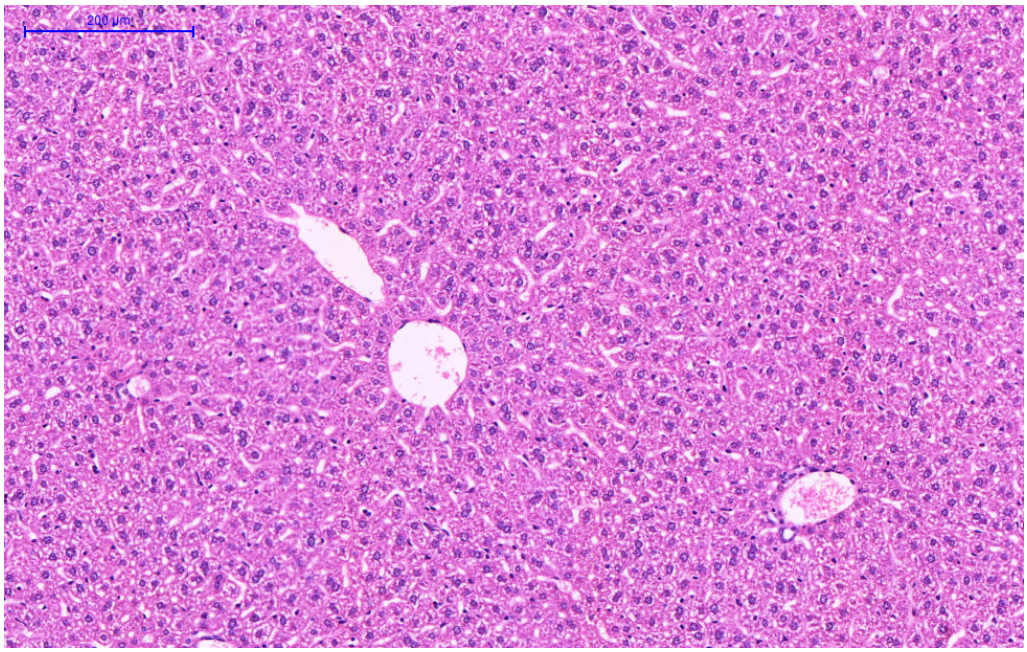
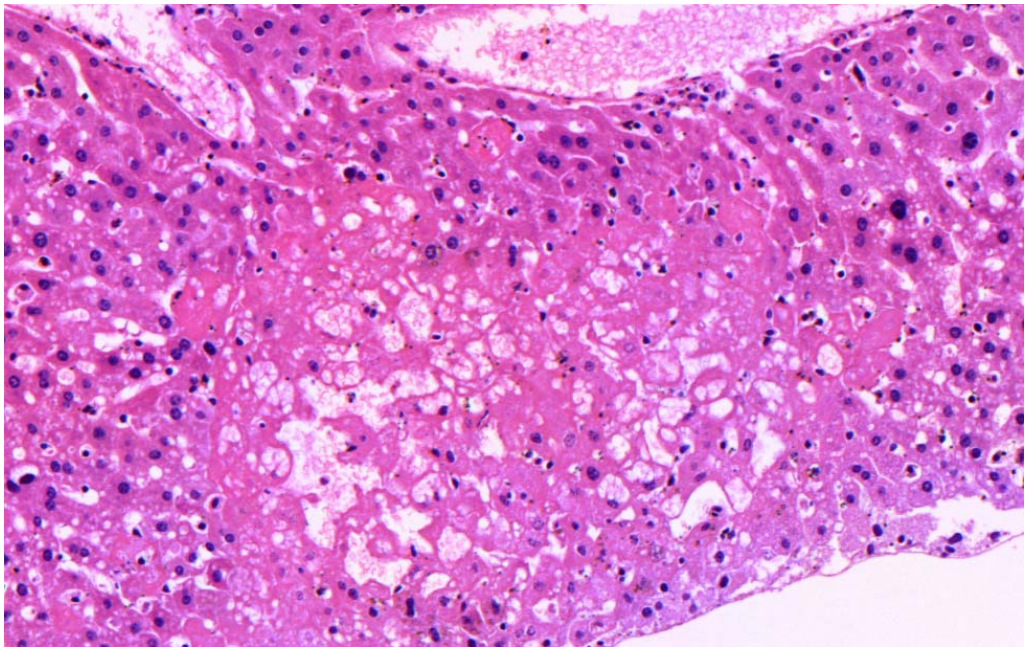
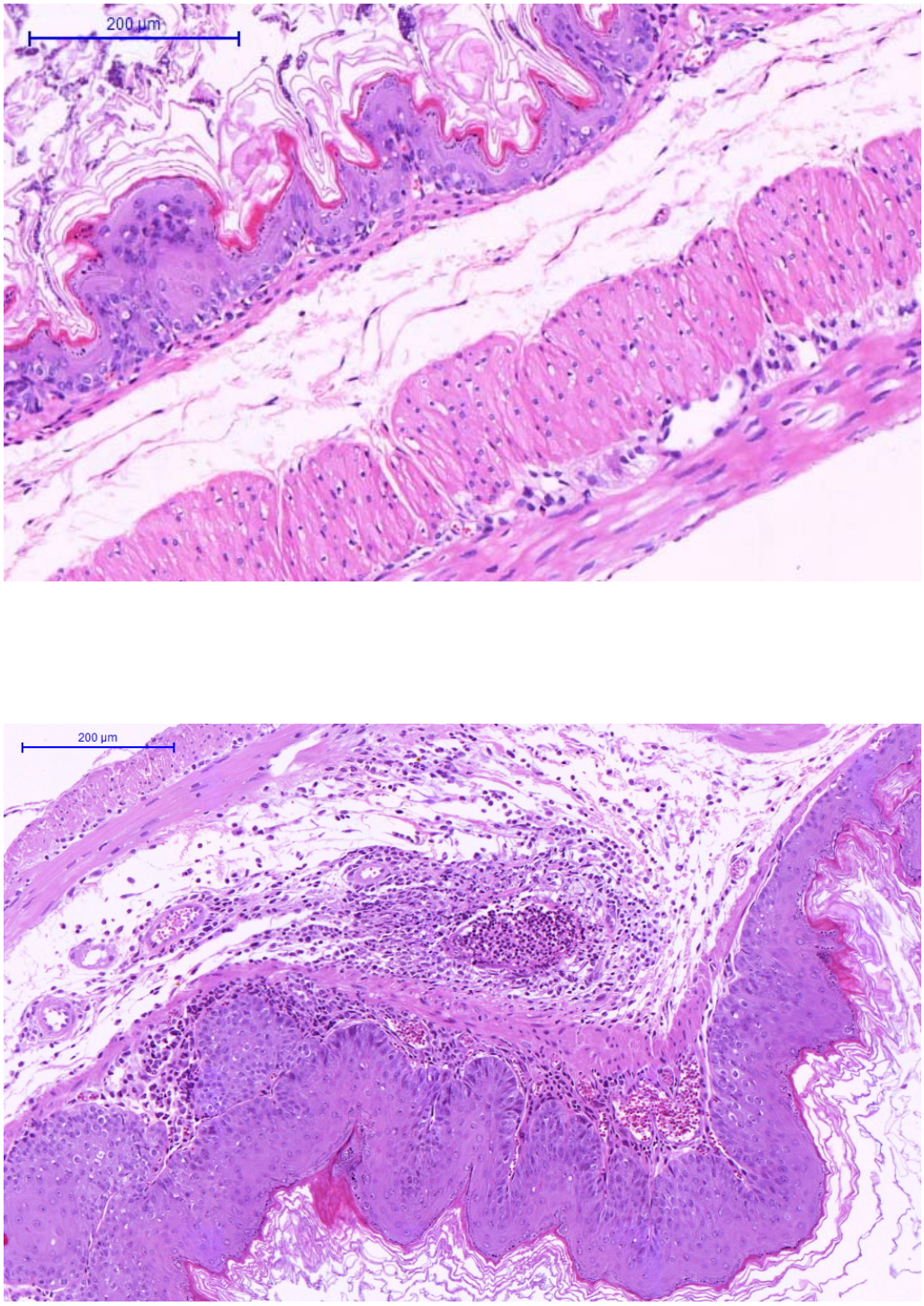


Figure 38: **Light micrographs of liver from mice of *Experiment 1*. PLTX 180 µg/kg Moderate foci of hepatic parenchymal necrosis. Magnification 20x. (hematoxylin–eosin stain).**



During *Experiment 2*, no tissue changes were observed at lung, cardiac or spleen level. The only tissue changes were observed in mice administered with palytoxin at the dose of 30 µg/kg/day sacrificed on day 8. In particular, liver showed a decreased glycogen content, similar to that recorded during *Experiment 1*. Moreover, minimal focal infiltrates in the submucosa layer of the non-glandular stomach of one mouse was recorded (Figure 39).

Figure 39: **Light micrographs of non-glandular stomach from mice of *Experiment 2*. Top; Control mouse; Bottom; PLTX treated mouse at 30 µg/kg/day. Magnification: 10 x; hematoxylin-eosin stain.**



TOXICITY OF PLAYTOXIN AND OKADAIC ACID ASSOCIATION AFTER REPEATED ORAL ADMINISTRATION

The toxic effects of combined doses of palytoxin and okadaic acid was investigated after daily oral administration in mice. To this aim, groups of 8 animals were administered by gavage with palytoxin for 7 days (3 or 10 µg/kg/day) in combination with 185 µg/kg/day of okadaic acid. The effects of combined toxins were compared to that of each toxin alone at the same doses.

Mortality and Clinical Signs

The daily administration of palytoxin and okadaic acid induced lethal effects only in two animals which received different doses of the blends of toxins: one mouse administered with 3 µg PLTX/kg/day and 185 µg OA/kg/day during d8, and one mouse treated with 30 µg PLTX/kg/day and 185 µg OA/kg/day during day 6.

Table 12: Incidence of mortality following repeated oral administration of PLTX and OA.

Groups (dose as µg/kg)	Study Day (number of animals)
control	(0/8)
PLTX 3	(0/8)
PLTX 10	(0/8)
PLTX 3 + OA 185	d8 (1/8)
PLTX 10 + OA 185	d6 (1/8)
OA 185	(0/8)

Mice administered with both the doses of palytoxin in association with okadasic acid and those administered with the higher dose of palytoxin (10 µg/kg/day) showed

episodes of scratching. In a limited number of animals (two) treated with PLTX at 10 µg/kg/day and OA (185 µg/kg/day), on day 6 and 7, respectively, exhibited rotation on themselves along the vertical axis, difficulties in simple mobility and showed abdominal breathing and gasping (Table 13).

Table 13: Detailed clinical signs of mice following seven days oral treatment with PLTX and OA.

	CONTROL	PLTX 3 µg/kg	PLTX 10 µg/kg	PLTX 3 µg/kg + OA 185 µg/kg	PLTX 10 µg/kg + OA 185 µg/kg	OA 185 µg/kg
Scratching	0	0	+	+	++	0
Reactivity to touch	0	0	0	0	0	0
Abdominal dilation	0	0	0	0	0	0
Hypersensitivity to light	0	0	0	0	0	0
Difficulties in mobility	0	0	0	0	+	0
Abdominal breathing/gasping	0	0	0	0	+	0

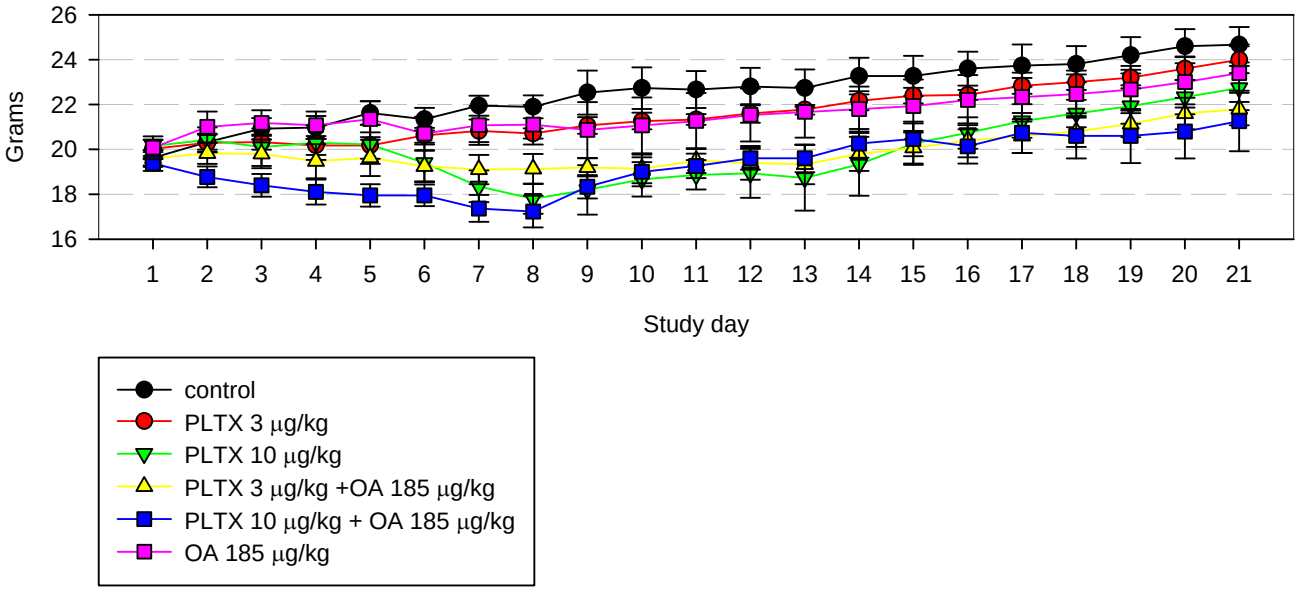
Legend: 0 within normal values; + increase frequency; ++ medium increase in frequency; +++ high increase in frequency; ++++ highly increased frequency

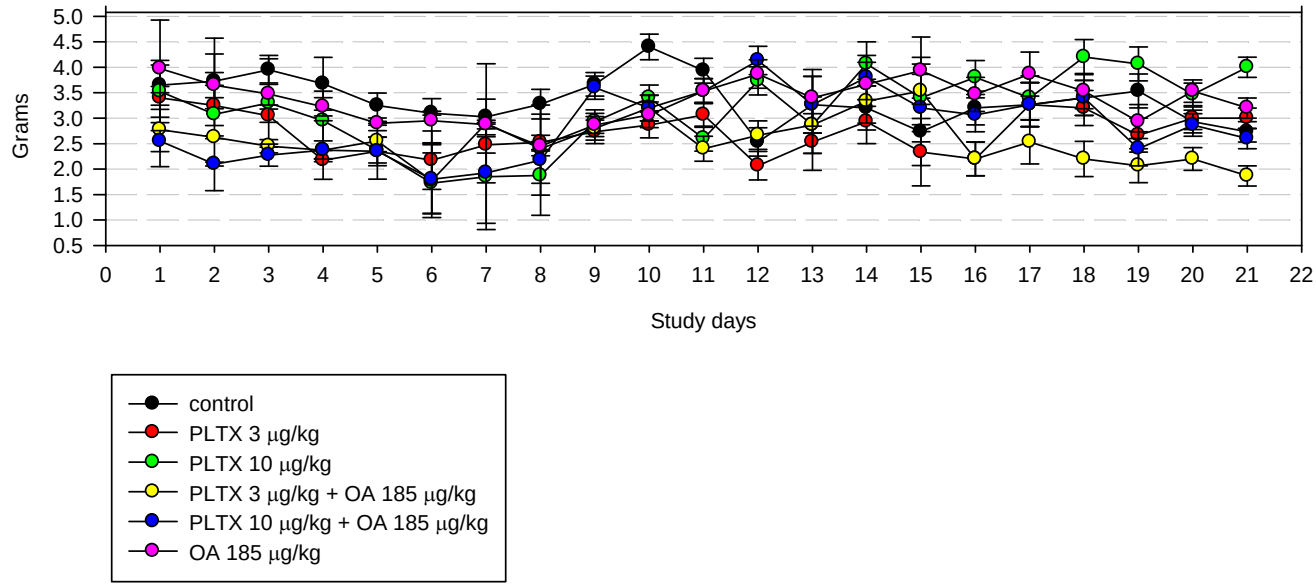
Food intake and body weight

Following the first treatment, a slight decrease in body weight was noticed for the groups treated with PLTX 3 and 10 µg/kg in combination with OA which persisted throughout the treatment phase, associated with a slight decrease in food consumption. Upon cease of treatment, the trend towards a recovery was evident in both parameters. Animals dosed with PLTX alone at a dose of 10 µg/kg, PLTX 10 µg/kg in combination with OA 185 µg/kg and PLTX 3 µg/kg in combination with OA 185 µg/kg did not reach

control comparable value at the end of the recovery period. Other groups had no significant differences from control through the entire study. Nevertheless, a maximum decrease in body weight of approximately 21% was the highest reached by the group dosed with PLTX 10 μg/kg in combination with OA 185 μg/kg on day 8 (first day of recovery).

Figure 40: **Body weight (upper panel) and food consumption (lower panel) following repeated oral administration for 7 days plus 14 days off treatment (mean ± SD per group).**





Gross pathology and organ weight

No treatment related differences, with respect to controls, was observed at gross pathology examination.

Decrease in liver absolute weight was recorded in all dosed animals at the acute sacrifice, in particular the highest animals given PLTX 10 µg/kg alone or in combination with OA; seemingly the presence of OA enhanced absolute liver weight decrement. A similar trend was noted for kidney weight, but to a lesser magnitude.

At the final kill even though a trend towards recovery was recorded, liver weights of the dosed groups remained lower with respect to the concurrent value for the controls, in particular at PLTX 3 or 10 µg/kg in combination with OA.

The overall renal weight was higher in a non dose dependant manner in all treated animals, versus control.

Mean absolute spleen weight resulted comparable to the control at the acute sacrifice but decreased at all doses, especially PLTX 10 µg/kg in combination with OA or OA alone, with respect to the concurrent control values at the final kill.

Figure 41: **Absolute organ weights in mice on day 8 (top graph) and day 21 (bottom graph) after repeated oral administration of palytoxin and okadaic acid (mean ± SD).**

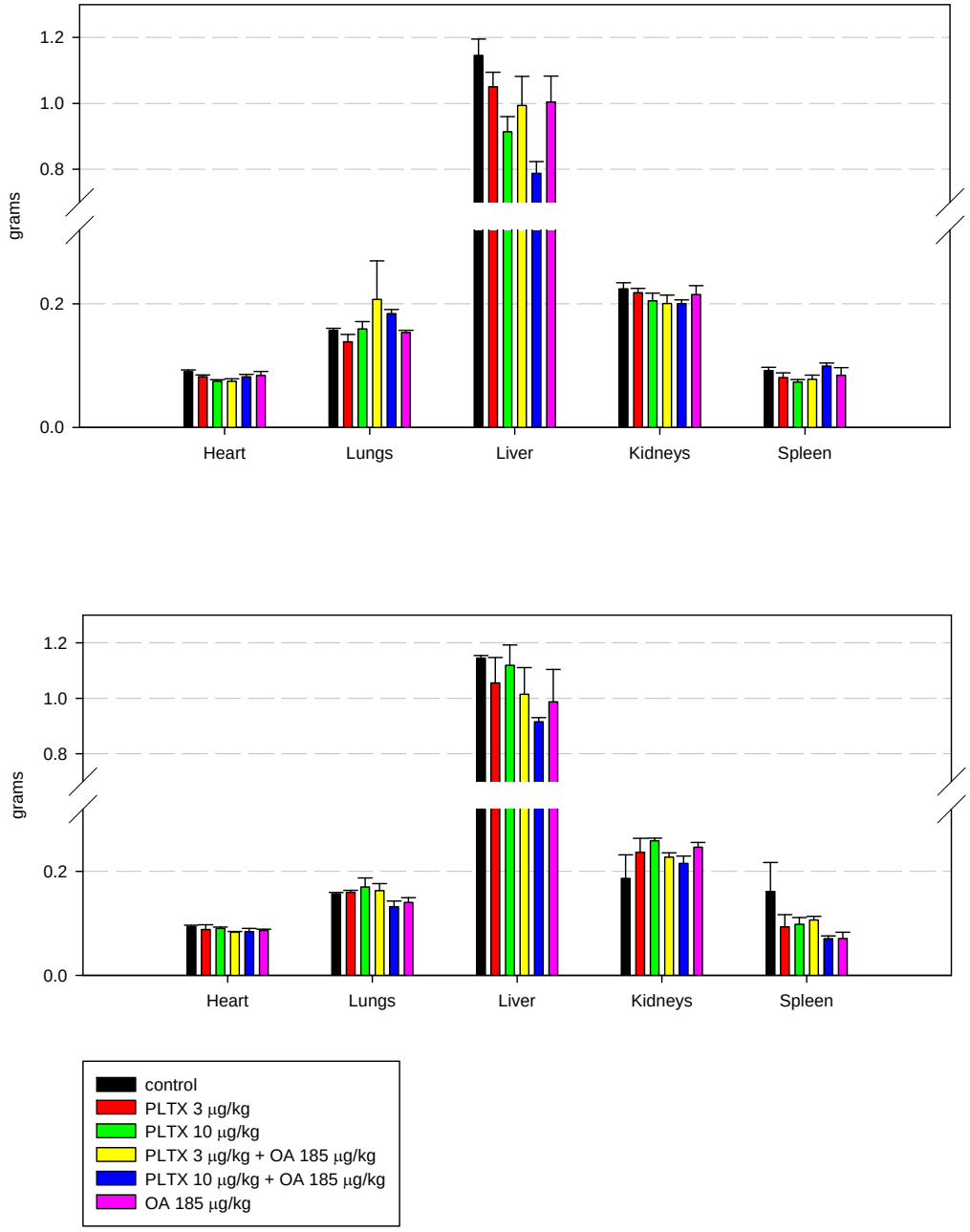
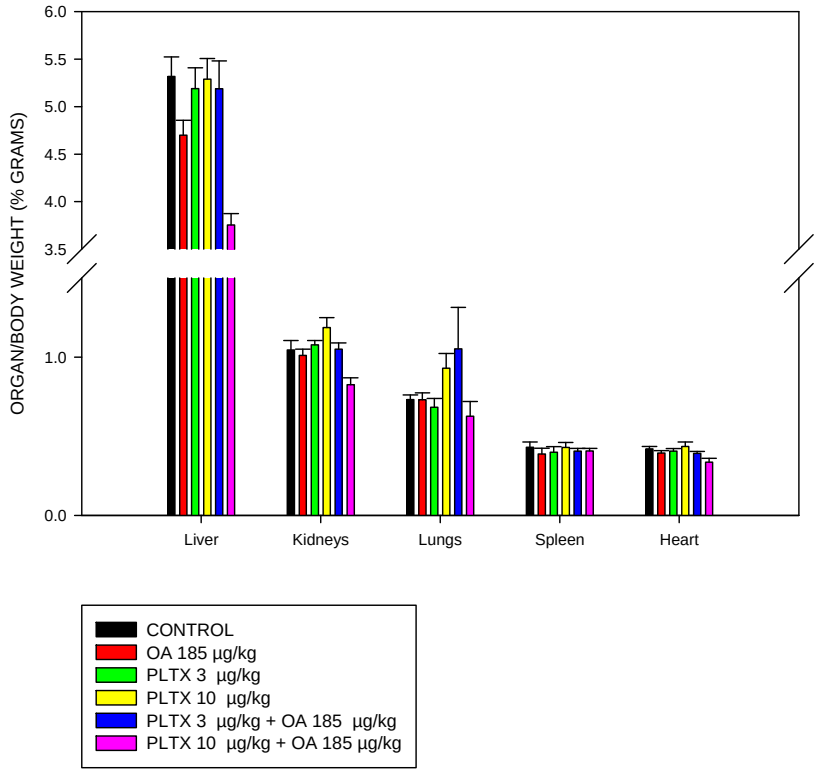
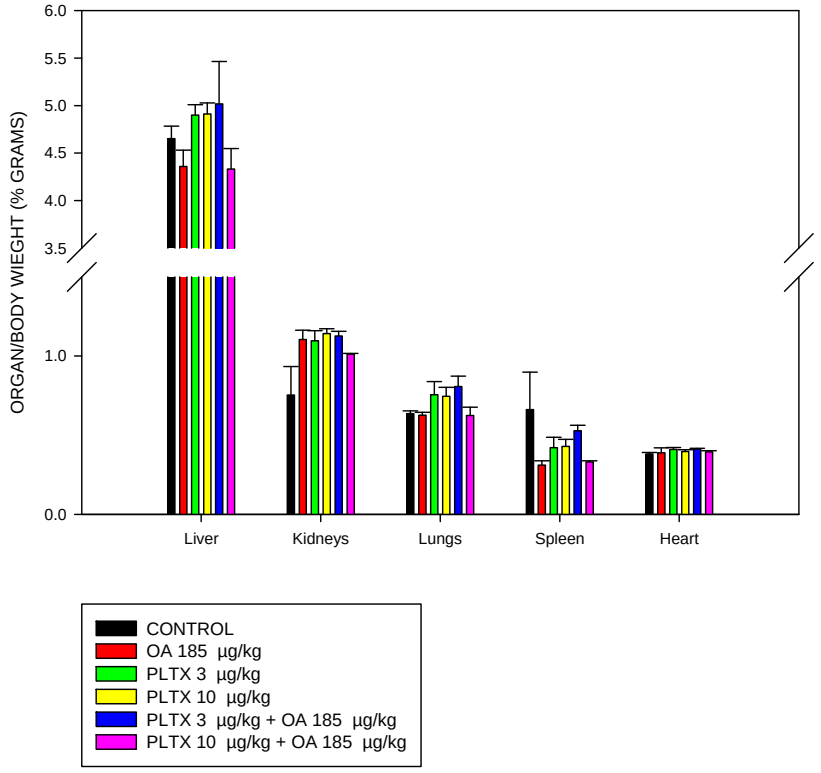


Figure 42: **Relative organ to body weight ratio weights in mice on day 8 (top graph) and day 21 (bottom graph) after repeated oral administration of palytoxin and okadaic acid (mean ± SD).**



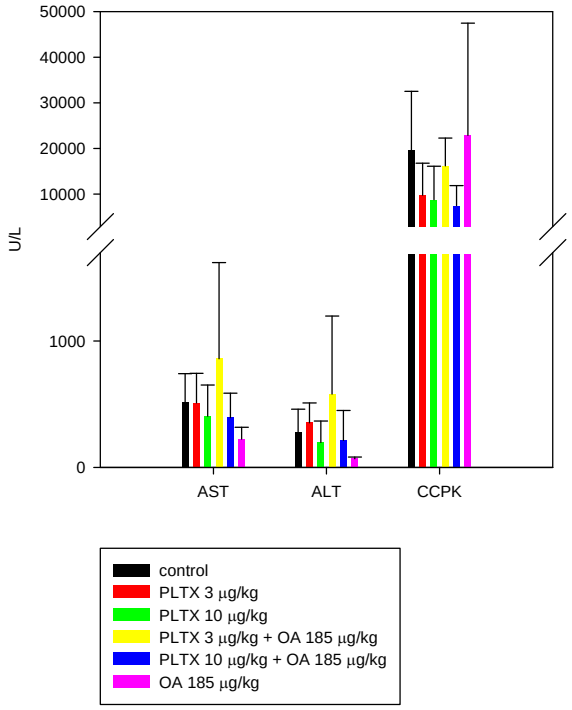


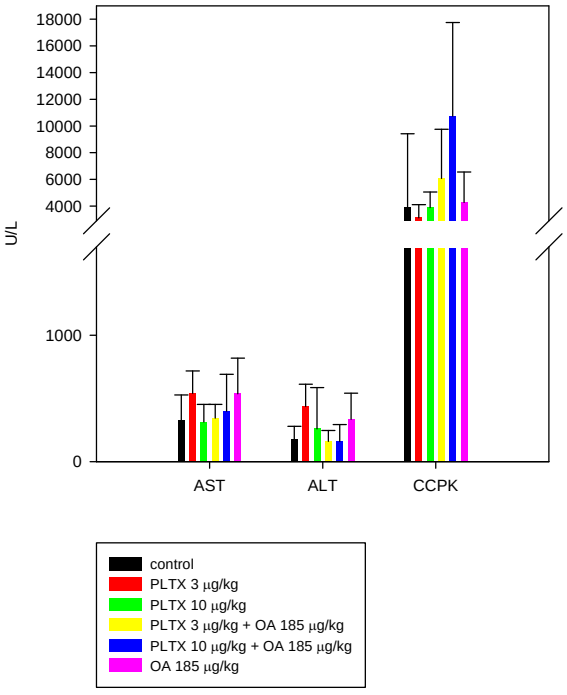
Clinical Chemistry

Combination treatment, in particular the 3 μg/kg PLTX with OA, caused an increase in hepatic enzymes (AST and ALT) which trended towards a recovery in animals sampled following the non treatment period. Slight elevations in ALT and AST were also noted in almost all dosed groups at the end of the withdrawal period.

The 3 μg/kg PLTX with OA group, showed increased CCPK, but the variability of the values did not differ from those observed for the control animals. Increase in CCPK was also observed in animals treated with OA alone, again returning to values similar to controls during the off treatment period. Inter-individual variability was evident in all groups for each parameter.

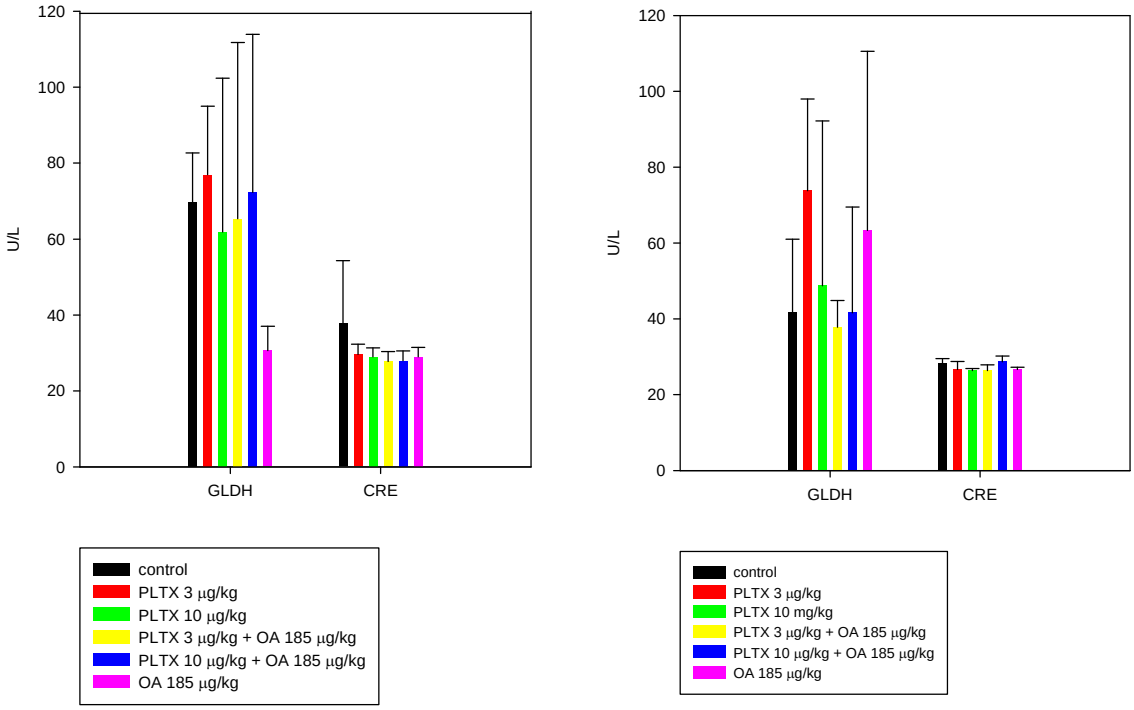
Figure 43: **Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine kinase phosphate (CCPK) in mice at 24 h after daily repeated oral administration of palytoxin and okadaic acid (day 8; top graph) or after the recovery period (day 21; bottom graph) (mean ± SD).**





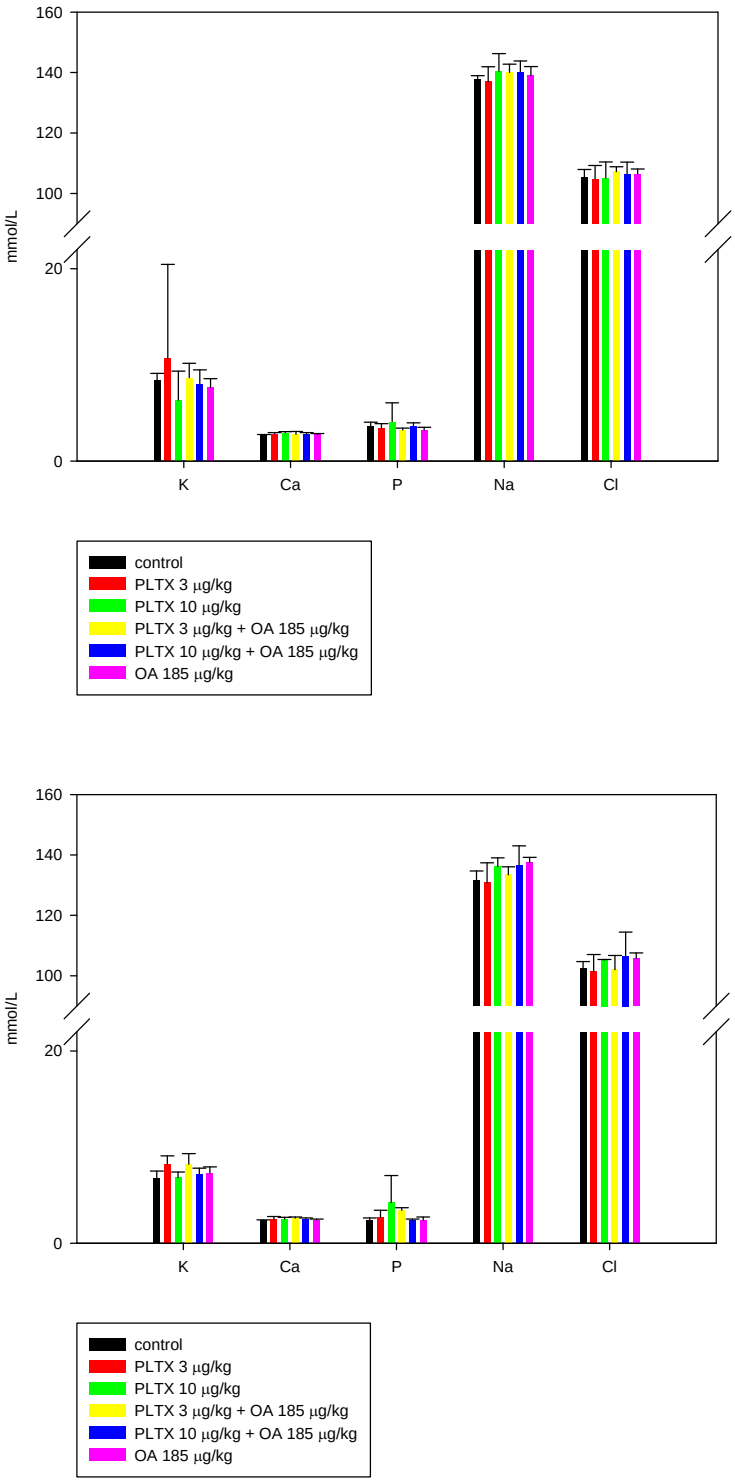
Slight decrease with respect to controls was observed for creatinine at the acute sacrifice time point (23 hours after the last treatment). No significant changes were recorded after the recovery period. GLDH values remained comparable to controls at the acute sacrifice even though there was a high inter-individual variability. Increase in almost all dosed animals was registered at the final kill.

Figure 44: **Serum levels of Glutamate dehydrogenas (GLDH) and creatinine (CRE) in mice at 24 h after daily repeated oral administration of palytoxin and okadaic acid (day 8; left figure) or after the recovery period (day 21; left figure)(mean ± SD).**



K+ ion blood concentration showed an increase in animals dosed with 3 μg/kg PLTX alone or in combination with OA throughout the study, comparable to control values for the remaining dosed animals. No alterations for the remaining ions at the sacrifice 24 hours after the last treatment while a slight elevation in Na+ and Cl- was registered on the sacrifice after 14 days of recovery.

Figure 45: **Serum levels of Na^+, Ca^{2+}, inorganic phosphate (P), K^+ and Cl^- in mice at 24 h after daily repeated oral administration of palytoxin and okadaic acid day 8 top; day 21 bottom graph. (mean ± SD).**

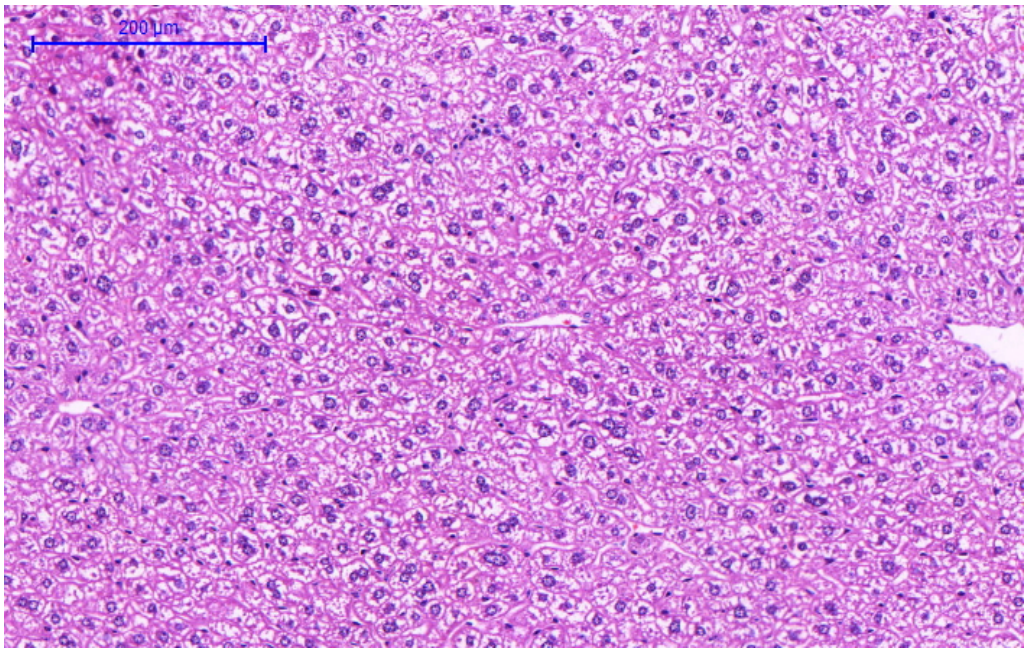


Light microscopy histological pathology

At the acute sacrifice findings were limited to one animal dosed with PLTX 10 µg/kg + OA 185 µg/kg which showed slight focal hepatic necrosis and moderate thymus atrophy. A single animal dosed with OA 185 µg/kg showed slight increase in vacuolization of the renal tubules.

No microscopic alterations were recorded at the final sacrifice.

Figure 46: **Light micrographs of hepatic parenchyma from a control mouse; hematoxylin-eosin stain. Scales – top 10x; bottom 20x.**



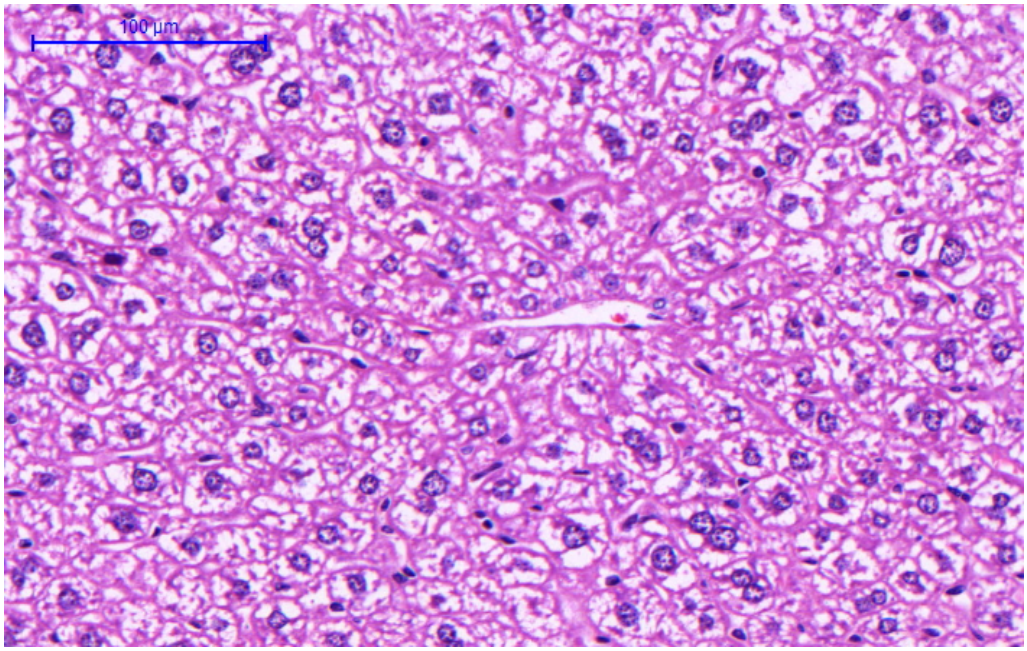
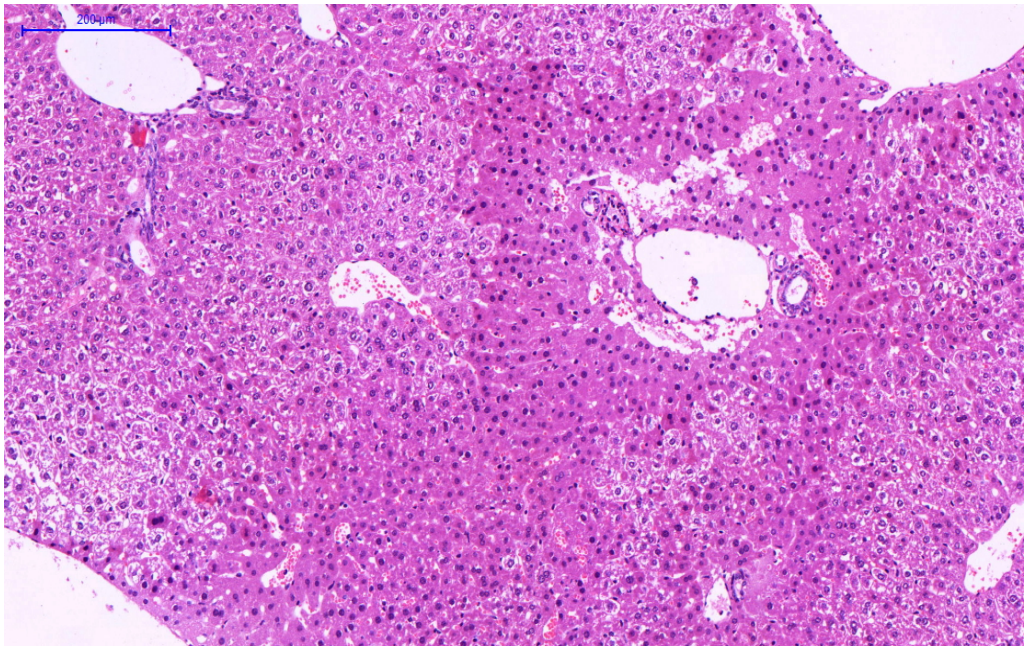


Figure 47: **Light micrographs of hepatc parenchyma from mice treated with repeated oral administration of palytoxin and okadaic acid of showing foci of hepatic necrosis; hematoxylin-eosin stain. Scales – top 10 x; bottom 20x.**



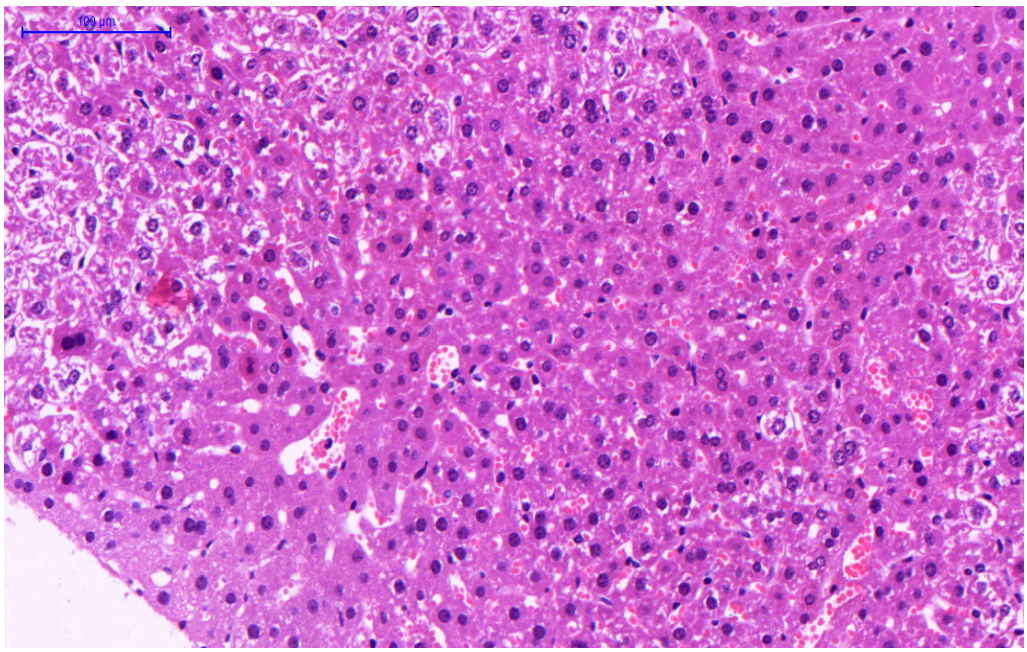
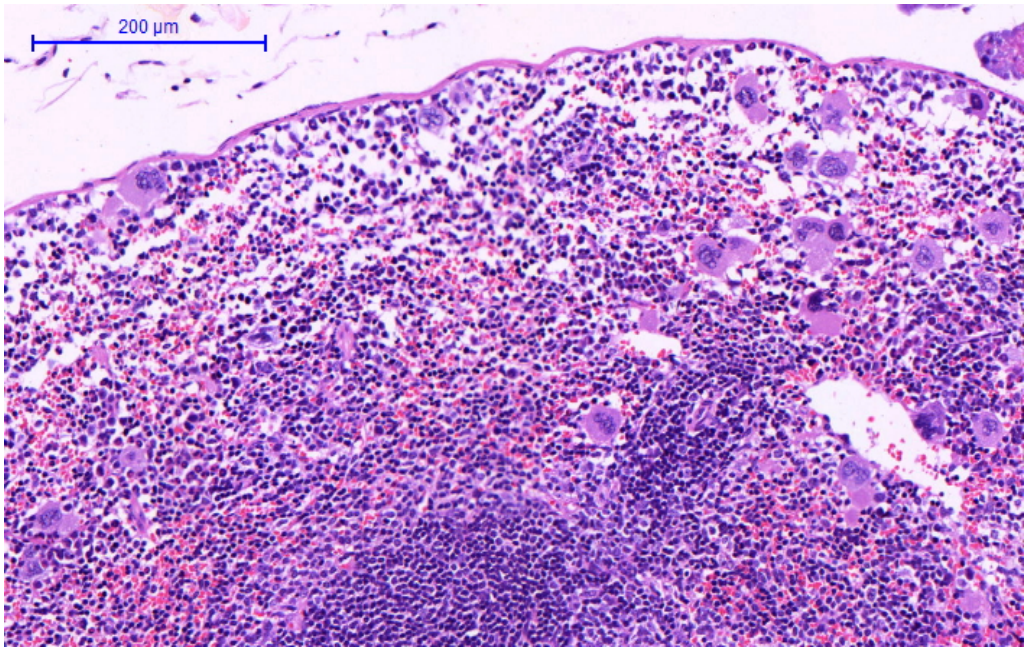


Figure 48: **Light micrographs of the thymus from a control mouse; hematoxylin-eosin stain. Scales – top 10x; bottom 20x.**



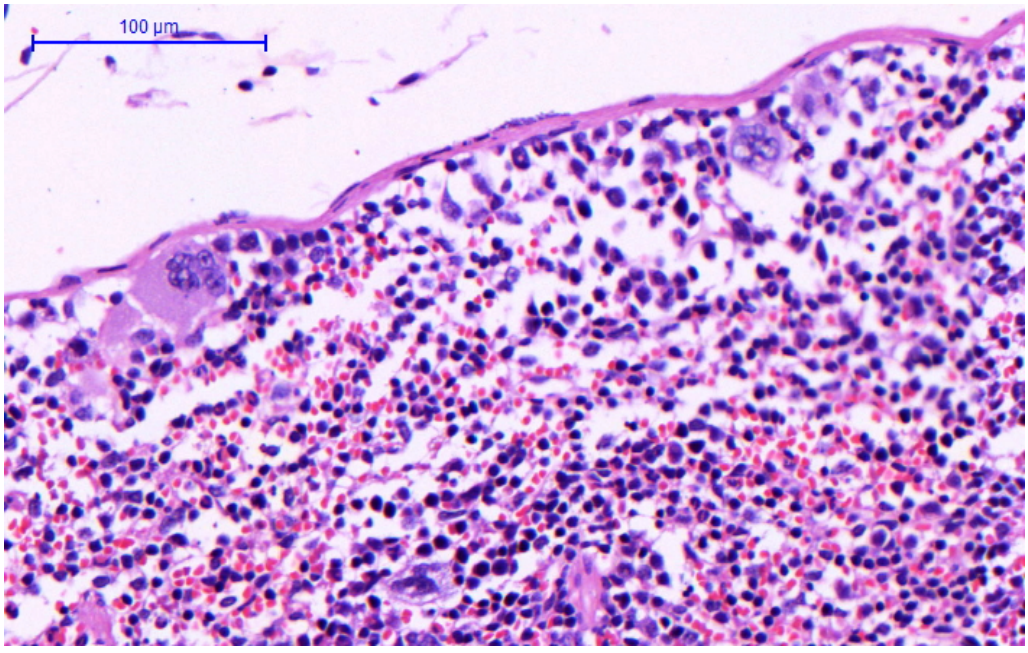
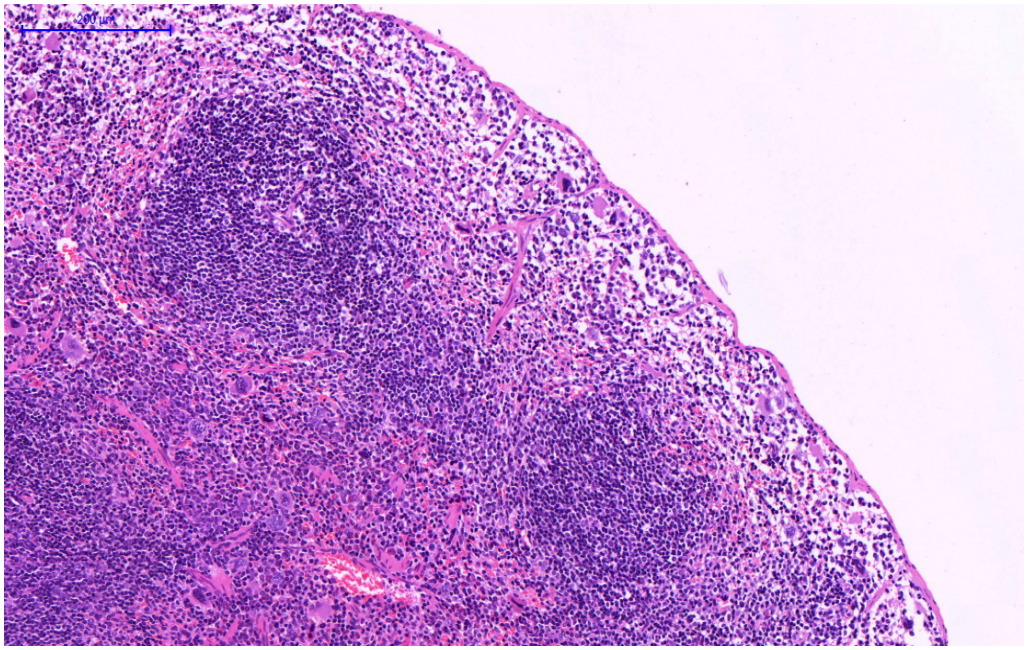


Figure 49: **Light micrographs of the thymus in a mouse treated with repeated oral administration of palytoxin and okadaic acid of showing foci thymic atrophy; hematoxylin-eosin stain. Scales – top 10x; bottom 20x.**



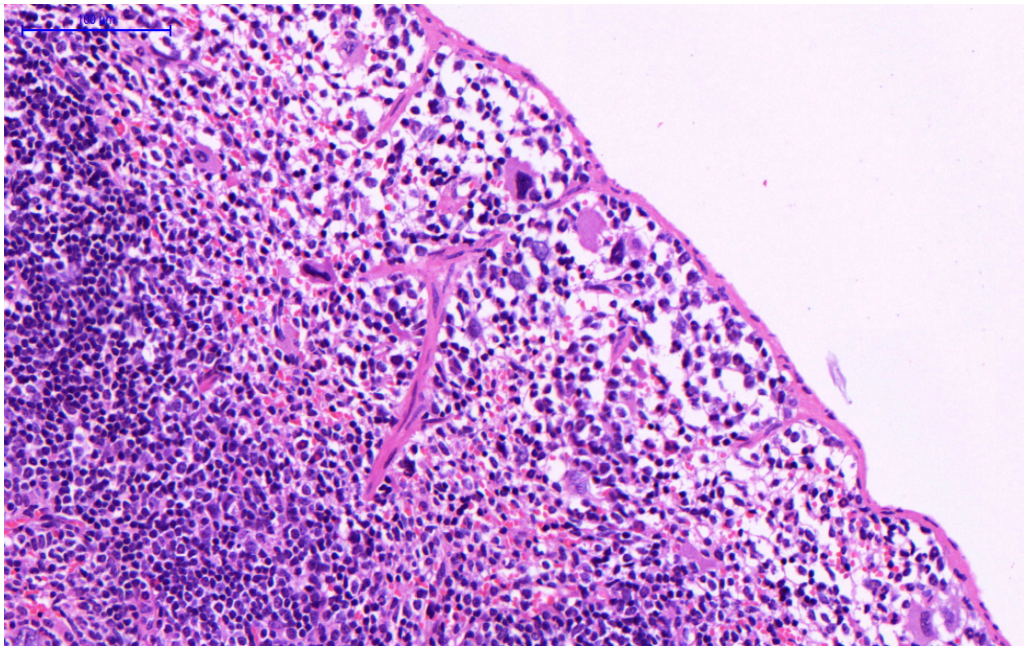
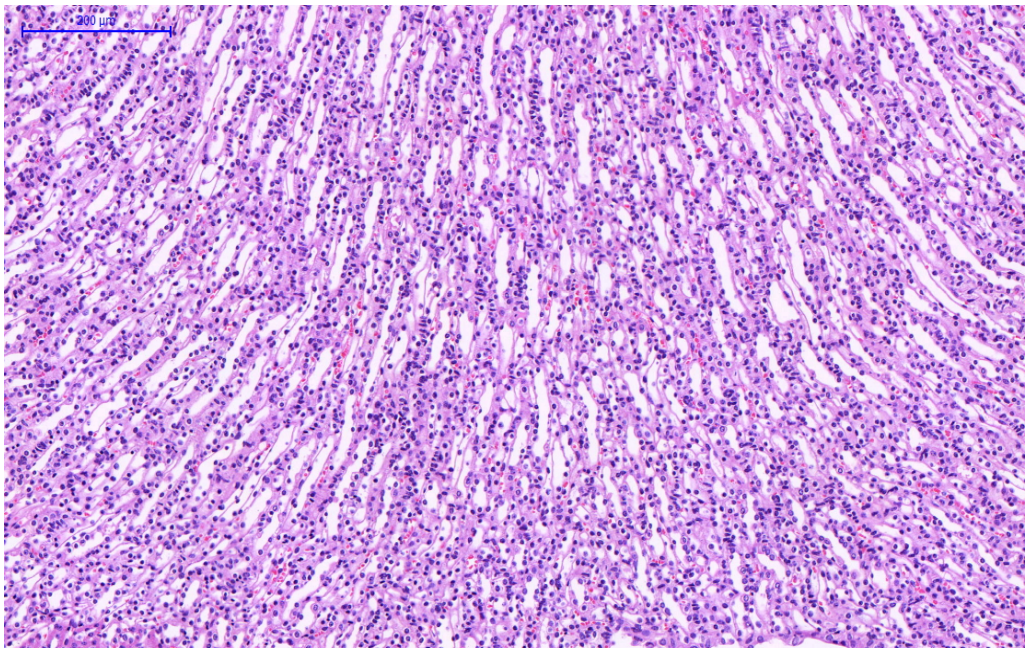


Figure 50: **Light micrographs of the kidney from a control mouse; hematoxylin-eosin stain. Scales – top 10x; bottom 20x.**



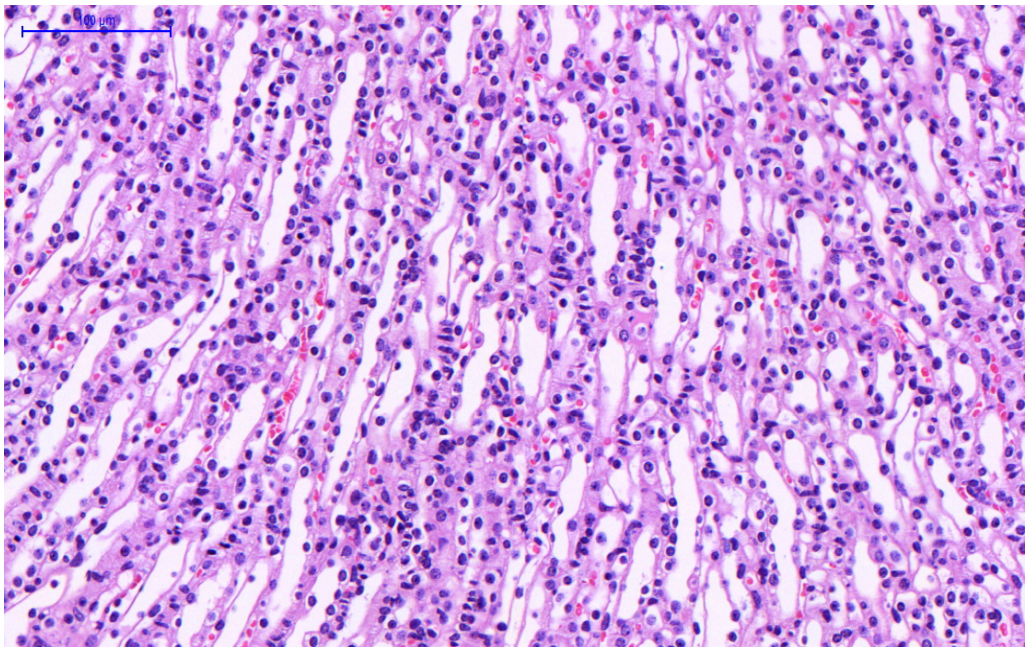
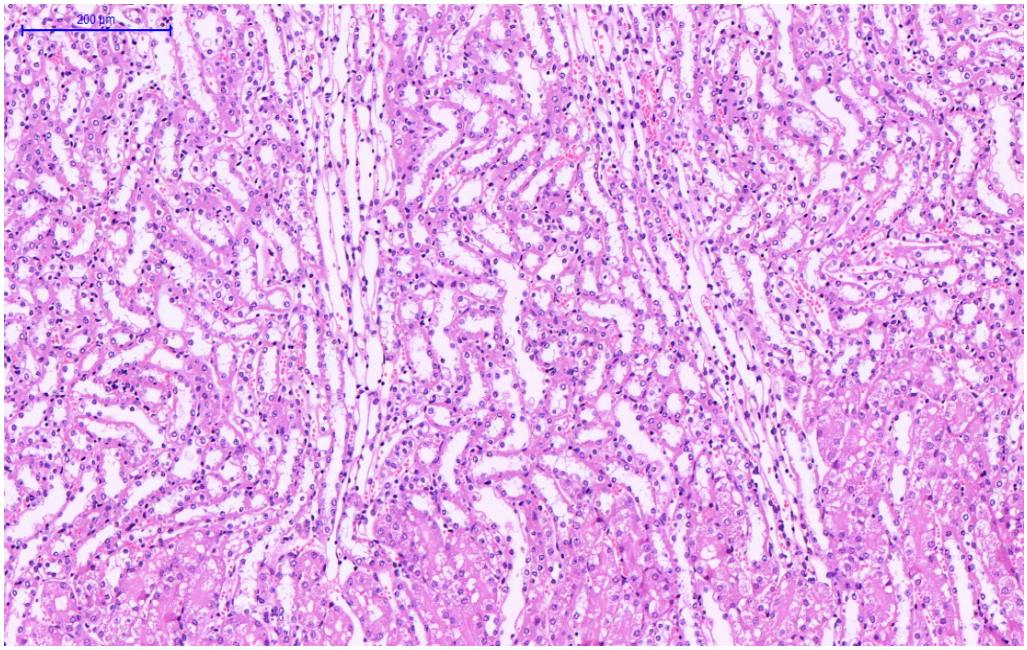
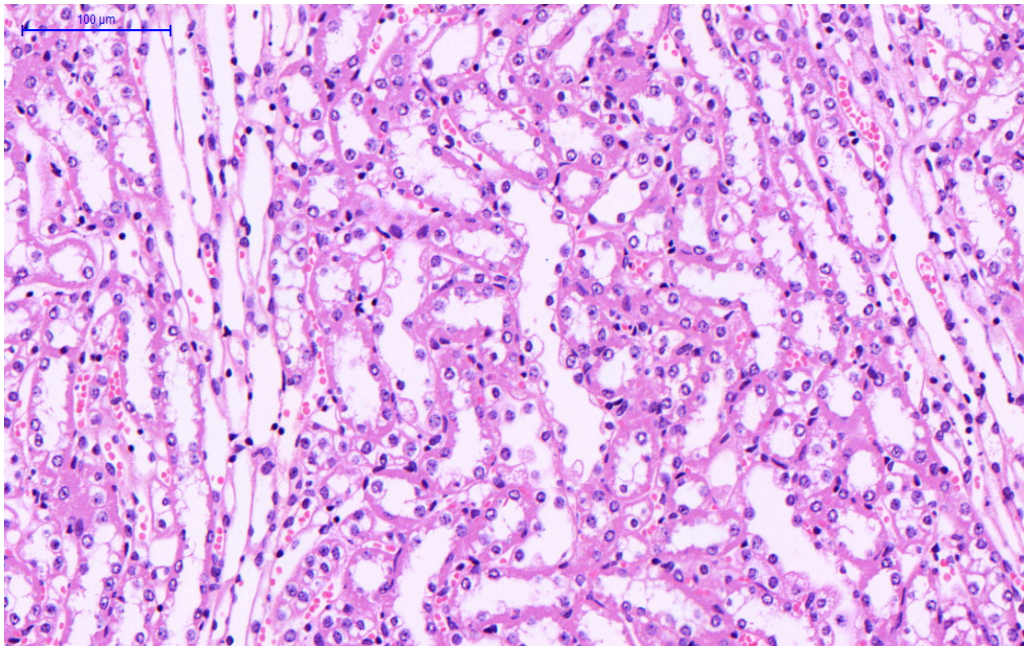


Figure 51: **Light micrographs of the renal tissue in a mouse treated with repeated oral administration of okadaic acid showing vacuolization of the tissue; hematoxylin-eosin stain. Scales – top 10x; bottom 20x.**





DISCUSSION

Shellfish toxicity is a burgeoning issue worldwide. Blooms of harmful algae are becoming increasingly more frequent and severe and non-indigenous toxigenic organisms have spread into many areas. These changes may depend on many factors such as climate change, growing levels of greenhouse gases in the atmosphere, eutrophication of coastal waters, reduced coastal biodiversity and transport of species from endemic to non-endemic areas in released ballast water, or transfer of shellfish stocks from one area to another. At the same time, more and more toxigenic algal species, toxin groups and congeners of known toxins are being found. It is thus crucial to have the right tools to assess the risks implied in shellfish toxicity.

Setting useful and meaningful thresholds and levels for daily intakes, which cover nowadays more important roles coupled with chemical shellfish toxin analytical methods are becoming accepted, robust data on acute oral toxicities are essential. Moreover, approved methods using certified standards are needed to establish the comparative oral toxicities of these toxins. One widely-accepted assumption, for which there is no corroborating data and that still has to be verified, is that the effects of the various congeners of the same toxin group are cumulative. Testing on combinations of toxin analogues is required to ascertain whether additive effects do actually occur. In rodent acute oral toxicity studies performed to date, administration has usually been by gavage. However, the structure of the rodent stomach and the consistency of its contents may lead to an artefact overestimation of acute toxicity. In fact, the Codex Alimentarius Commission recommended using administration in the diet instead of by gavage when studying the acute oral toxicity of shellfish toxins, to mimic more closely the human situation. In this case the animal feed has to be spiked with the test compound, and

completely consumed within a few seconds but problems of availability of algal toxins renders problematic this approach. Moreover, useful information would be provided from studies on the kinetics of toxins after treatment with a single oral dose.

Since people who regularly eat seafood may be exposed to low levels of shellfish toxins over long periods, it would be useful if regulatory bodies could establish TDIs for these substances. This would entail conducting repeat-dose feeding studies starting from 28-day studies, as suggested in OECD Guideline 407. Particular attention should be paid to chronic effects of toxins that have a slow elimination rate in mammals and may accumulate tissues, potentially causing severe toxic effects. The genotoxicity should be also tested using standard methods.

While risk assessment is normally carried out on young, healthy animals, in the human population aging or health issues may exacerbate the effects caused by some toxins. In the outbreaks, people who suffer from example from concomitant age related diseases, such as kidney disease, high blood pressure or diabetes, will react differently to toxin absorption and with the effects of the toxins alone or in combination. In order to induce visceral effects, absorption must occur in the gastrointestinal tract. The mucosal barrier, which consists of the mucus layer and the tight junction between mucosal cells, limits the absorption of toxins; gastrointestinal epithelium becomes thinner and more fragile frequently in old age and is in the same state in young adults and children. Intoxication in altered states could lead to different susceptibility in the exerted and observed effects. Toxicity could also be facilitated by epithelial damage to the stomach and intestine caused other concomitant factors such as alcohol or non-steroidal anti-inflammatory drugs.

Preliminary results obtained during the present work give further proof to the interpretation of results that must include evaluation of potential concomitant factors. In fact, PLTX and OA have been reported to be increasing frequently in marine edible organisms with the possibility of a simultaneous contamination and a consequent human co-exposure to these toxins through seafood consumption. Thus, it has been studied whether they influence the toxicity of each other when present in the body at the same time. Although the lethal dose for both toxins has been already determined by a number of studies, the aim of our study was to verify possible additive/synergic effects after acute and repeated oral exposure to the toxin blend, verifying also the toxicity of palytoxin alone after repeated oral administration, previously not investigated. To this aim, a single dose of OA was chosen on the base of previous pilot studies as the highest one where the clinical signs were reversible on a short term and diarrhoea was not debilitating the animals (370 μg/kg for the acute oral exposure or 185 μg/kg/day for the repeated exposure). Due to its intrinsic high potency, PLTX was administered at multiple doses, in order to obtain a wide range of dose-response starting from the very low doses of 30 μg/kg (acute oral exposure) or 3 μg/kg/day (repeated oral exposure).

The results of the acute oral toxicity study evidenced no lethal effects in mice administered with OA alone, whereas lethal effects were recorded starting from 90 μg/kg of PLTX, even in association with OA or as single toxin. Thus, lethal effects of PLTX alone occurred at doses lower than the lethal ones recorded by previous studies [85]. Moreover, after co-administration of PLTX (90 and 270 μg/kg) and OA (370 μg/kg) almost all the mice showed scratching and, prior to death, the signs of toxicity were similar to those observed after the administration of the corresponding doses of PLTX alone, but were amplified by the co-administration of OA which suggest an additive

effect between the two toxins. An indication of an additive effect is suggested also by the necropsy observations: 24 h after the toxins administration revealed fluid accumulation and redness in the small intestine of mice treated with all the doses of PLTX combined to OA, with a frequency higher than that recorded in mice treated with OA (fluid accumulation) or PLTX (redness of the intestinal wall) alone. Similarly, histology revealed that oral co-administration of PLTX and OA caused changes at gastric level. Tissue changes were noted in animals sacrificed 24 h after administration and no alterations were recorded in mice after the withdrawal period of 14 days. In particular, slight to mild ulcers accompanied by inflammatory infiltrations of the forestomach was observed in only 2/5 mice dosed with 90 μg PLTX/kg together with 370 μg OA/kg and in 2/5 mice administered with 270 μg PLTX/kg associated to 370 μg OA/kg. Moreover, slight inflammatory infiltrates typical of gastritis was observed in the forestomach of 2/5 animals administered with the highest dose of PLTX combined with OA. At the hepatic level, a decrease of glycogen content in hepatocytes was observed in almost all animals given the highest PLTX dose (270 μg/kg), alone or in combination with OA (370 μg/kg). Tissues alterations at gastric and hepatic level were previously observed after acute oral administration of higher doses of PLTX (424 or 600 μg/kg, respectively) in mice that survived after the toxin administration and sacrificed after 24 h [85].

Prior to verify the effects of the two toxins after repeated oral exposure, a toxicity study on PLTX alone was carried out as no short-term studies on this toxin were previously carried out. The study revealed that daily oral administration of PLTX, for 7 days, induced lethal effects starting from the dose of 30 μg/kg/day, more than 10 times lower than the lethal dose recorded after acute oral administration [85, 86, 87]. In some mice death occurred at the third day of treatment (90 and 180 μg/kg/day) or at the fourth day

(30 μg/kg/day) and lethality was also observed during the 14-days recovery period. This indicates that the toxic effects induced by repeated exposure to the toxin are not completely reversible and can progress causing the death of animals. No lethal effects or other signs of toxicity were recorded at the lower administered dose (3 μg/kg/day).

Signs and symptoms of animals, such as paralysis of the hind limbs, loss of the righting reflex and breathing difficulties, suggest a neuromuscular action of PLTX and/or its metabolites. Moreover, the recurrence of these observations in different studies after intraperitoneal [98, 99] or oral acute administration [27, 85, 86] suggests these signs as a specific effect of the toxin.

A significant decrease of body weight was observed at the dose of 30 μg/kg/day and above that can be related to the worsening of animals' conditions after toxin administration, as well as to a reduced food intake, which is in turn associated with the observed decrease of glycogen content in hepatocytes. However, reduction of glycogen content in hepatocytes was not accompanied by a reduced liver/body weight ratio. On the contrary, at the dose of 30 μg/kg/day, serum level of some enzymes markers of liver injury (transaminases and glutamate dehydrogenase) were significantly increased. Similarly, significantly increased levels of transaminases were recorded in mice after acute oral administration of considerably higher doses of PLTX or 42-hydroxy-PLTX [85, 86].

Swelling of the abdomen was observed at doses equal or higher than 30 μg/kg/day. It was probably related to mucous fluid and gas accumulation in the gastrointestinal lumen, observed during necropsy, which suggests an altered functionality or absorption at this level. The latter could at least in part explain the decreased body weight of animals, more evident before death. Despite macroscopic changes at gastrointestinal level, findings

by histological analysis were limited to the forestomach (erosion or minimal focal infiltrates in the submucosal layer). It has to be noted that slight gastric and small intestine injuries have been previously noted after single oral administration of PLTX (500 μg/kg)[87]. In addition, inflammation at forestomach was previously recorded in mice after 24 h from the acute oral administration of PLTX or 42-hydroxy-PLTX, starting from the dose of 424 μg/kg [85, 86]. Nevetheless, the toxic effects at forestomach, typical of rodents, are considered as not predictive for toxicity in humans.

Other organs affected by the repeated administration of PLTX were the lungs, where alveolar edema, occasionally associated to acute inflammation and necrosis, had been recorded at doses ≥ 30 μg/kg/day. These changes were noted mainly in mice dead before the end of the treatment period, possibly associated to the observed breathing difficulties. The lungs as targets for palytoxin were previously noted not only after intra-tracheal instillation of PLTX but also after oral and sublingual administration. In particular, in the latter case, interstitial inflammation, bleeding, edema and alveolar destruction were described [87]. This suggests the lungs as sensitive targets for PLTX and that an effect at this level could be involved in the severe breathing difficulties observed in mice prior to death.

The skeletal muscle is also considered a sensitive target organ for PLTXs, on the basis of epidemiological data [93, 94, 96]. However, no specific morphological skeletal muscle alterations were noted by light microscopy, but animal signs or symptoms, including as paralysis and loss of the righting reflex, do not rule out a possible effect at this level. Other findings (including depletion of the lymphoid elements of the lymphatic follicles or increased relative kidney weight) are spotted, not dose-related and appear to be not relevant for the overall toxic effects.

Thus, the daily oral administration of palytoxin to mice resulted in a provisional NOAEL of 3 μg/kg/day, with lungs, heart, liver and gastrointestinal tract as the main targets. This dose level was subsequently considered for the subsequent toxicity studies to evaluate the effects of combined doses of PLTX and OA after daily repeated oral administration.

Following repeated administration of combined doses of PLTX (3 and 10 μg/kg/day) and OA (185 μg/kg/day) lethal effects were recorded on days 8 and 6 in two animals administered with the toxins blend at the doses of 3 and 10 μg/kg/day of PLTX combined with OA (185 μg/kg/day), respectively.

Recurring clinical signs were recorded in all groups dosed with the toxin blend, and were super-imposable to those observed *a priori* and were most likely attributable to the presence of PLTX rather than of OA. These were mainly scratching, limb paralysis and piloerection. No alterations were noted in mice dosed with PLTX alone and only a transient slight diarrhoea was recorded in one animal administered with OA alone. In an overall clinical assessment, slight additional effects between toxins could not be completely ruled out. Body weights resulted reduced in mice administered with the toxins blend and in mice administered with PLTX alone at the dose of 10 μg/kg/day. Mice administered with the highest dose of PLTX (10 μg/kg/day) combined with OA (185 μg/kg/day) resulted markedly affected, showing a body weight loss of about 21 %. During the withdrawal period of the study (up to 14 days after the last treatment) a trend toward recovery was noted among the various groups even though the final body weight was in general lower than in control animals. Food consumption inevitably varied accordingly to the body weight trend.

No alterations occurred in any of the animals during the gross pathology examinations at both scheduled times of sacrifice (24 h and 14 days after the last treatment). Clinical chemistry highlighted minimal changes in a limited number of parameters assayed: after 24 h from the last treatment, transaminases increase were observed, which during the off treatment period returned to values comparable to controls. Microscopically, in one animal given PLTX (10 μg/kg/day) associated to OA (185 μg/kg/day), slight foci of hepatic necrosis accompanied by moderate atrophy of the thymus were noted at the acute kill. Moreover, a single animal dosed with OA (185 μg/kg/day) showed slight increase in vacuolization of the renal tubules at the same scheduled sacrifice. No histological alterations were registered after 14 days from the end of the treatment.

The focal necrosis of the liver observed in one mouse was not associated to a significant increase in AST, ALT or GLDH values, which were nevertheless high as standalones (492, 125 and 113.6 U/L respectively), but did not differ from other individual values observed at that time point for this and other doses. In addition, creatinine serum levels, as index of renal functionality, were not increased in the animal dosed with OA alone which showed slight vacuolization of the renal tubules.

In conclusion, the results obtained with the toxins alone completely met our expectations based on the previous data, confirming the scope of the study. Animals treated with doses near to sub-lethal doses of PLTX, OA or a blend of the two toxins did not show univocal clinical and microscopic alterations or alterations of selected clinical chemistry parameters. Anyway, the toxin(s) affected in a wholesome manner the health of mice as evidenced by the reductions in body weight and food consumption, associated in certain cases with overall clinical wellbeing. Furthermore, the 14 days withdrawal period

was not enough to completely restore health conditions in mice after a repeated toxin exposure. Moreover, the toxins blend caused two dose related sudden fatalities after repeated administration reinforcing the hypothesis of combined toxic action(s) even though no specific toxin related alteration of animal behaviour could be univocally addressed or identified.

The latter study, overall, highlighted that the concomitant presence of two marine toxins could increase their toxicity profile and latent effects although no evident synergic or additive effects were observed, even if they can not be ruled out.

The next steps would include a confirmatory study in a larger group of animals followed by a 14- and 28-day repeated dose study. Additional studies which may be useful in evaluating 'true' and realistic conditions of intoxications should also include animal studies aimed to investigate the impact of age and of impairment of the gastrointestinal tract on the toxicity of shellfish toxins.

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