RISK ASSESSMENT OF HUMAN CONSUMPTION OF POTENTIALLY CONTAMINATED RED SWAMP CRAYFISH (*PROCAMBARUS CLARKII*): A CONCEPTUAL APPROACH

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RESUMO

Pretende-se aprofundar o conceito da análise de risco inerente ao consumo humano de crustáceos contaminados com xenobióticos. Deu-se particular importância à análise do consumo do lagostim vermelho da Louisiana (*Procambarus clarkii*), espécie invasora não indígena que nos últimos anos assumiu um inusitado destaque no âmbito ambiental e gastronómico Português. Compilaram-se dados disponíveis na literatura sobre a possível contaminação deste organismo, no sentido de permitir uma avaliação ecotoxicológica e estabelecer uma ligação entre a contaminação deste recurso e efeitos potenciais toxicológicos em humanos.

PALAVRAS-CHAVE

Lagostim vermelho da Louisiana, *Procambarus clarkii*, análise de risco, contaminação, avaliação toxicológica

ABSTRACT

Our goal was to deepen the concept of risk assessment associated to human consumption of crustacean species contaminated by anthropogenic xenobiotics. Consumption of red swamp crawfish (*Procambarus clarkii*), an invasive species that has recently assumed a leading role in Portugal, both environmentally and gastronomically, is emphasized. Data was compiled from published literature, concerning its possible contamination, in order to allow an ecotoxicological assessment and to establish a link between contamination of this resource and toxicological potential effects on humans.

KEYWORDS

Louisiana's Red Crawfish, *Procambarus clarkii*, risk assessment, contamination, toxicological evaluation

1. **INTRODUCTION**

The red swamp crayfish (*Procambarus clarkii*) is a crustacean from the North-American state of Louisiana, and also from the north of Mexico (Barbaresi and Gherardi, 2000). This species was introduced in several other locations, and Europe was not an exception. According to Anastácio (1993), the Iberian Peninsula is a particular location where this species found favorable conditions. Due to (i) its high breeding capacity (about 500 eggs in each reproductive cycle, with 3 cycles per year) (Mc Clain *et al.*, 2005), (ii) the protection of eggs by the mother, and (iii) its intrinsic resistance to adverse conditions (nutrients, temperature, predators), *P. clarkii* is an extremely persistent species. Besides its persistency, it exerts deleterious effects on ecosystems due to predation (Kerby *et al.*, 2005; Correia, 2002), spreading of fungal and viral infections, and degradation of the river's bottom by burial activity. Owing to its adaptability, this species has been found in a large number of geographical locations, such as the United States of America (Louisiana) and Northeast of Mexico (Barbaresi and Gherardi, 2000); outside the American continent, it has been found in Spain, Portugal, Cyprus, France, Germany, Italy and the Netherlands (Anastácio, 1993). Moreover, this species spread to another locations, and nowadays it can be found worldwide, with the exception of Australia and Antarctica. The use of *P. clarkii* in human gastronomy is a cultural issue in the Mississipi Delta (Louisiana) and in Texas (U.S.A). Outside the U.S.A., it is a highly consumed crustacean species, mainly in locations where this type of food resources are not abundant (e.g. Scandinavia and Central Europe). It is also captured and consumed in specific areas of Portugal. Since it is used in human nutrition, and can potentially bioaccumulate heavy metals and pesticide residues, it is important to quantify the toxicological risk posed by its consumption. Several locations where *P. clarkii* has been introduced are subjected to continuous chemical contamination, due to diffuse domestic pollution, industrial discharges and agricultural runoff. Among the most important Portuguese locations where we can find this crustacean species, the Pateira de Fermentelos is an excellent example, since several studies refer the increase of chemical contamination of its waters by the mentioned sources (Teles *et al.*, 2006; Maria *et al.*, 2005). Several classes of anthropogenic contaminants can be found in this freshwater ecosystem, but pesticides and heavy metals assume a particular role. The commercial formulations Milraz® (cymoxanil and propineb) and Roundup® (glyphosate) are extensively used in agricultural activities (information given by local farmer associations), and the metals lead and cadmium have been identified in the waters of Pateira de Fermentelos and adjacent tributaries (Teles *et al.*, 2006). Several researchers (Rodrigues et al., 2006) questioned the use and presence of pesticides in water as it can result in damage, not only to the ecosystem itself, but also to human health (Feigenbrugel et al., 2005). Pesticides may exert deleterious effects at several levels, including damage to the nervous system, immunosupression, endocrine disruption, or onset of carcinogenesis (Caldas *et al.*, 2000).

1.1. **CYMOXANIL**

Cymoxanil is a fungicidal compound, and is one of the most used pesticides in Portugal. It acts through the inhibition of sporulation, and is active against pathogenic species of the *Perenosporales* order (FAO, 2005). It is extensively metabolized in animal models, such as the rat (EXTOXNET, 1997), and is rapidly eliminated via urine or faeces (about 85% is excreted within 48 hours following administration). After 96 hours, less than 1% of the administered dose remained in the tissues. The major excretory products were polar metabolites such as 2-cyano-2-methoxyimino acetic acid, glycine and other amino acid conjugates. These

metabolites are rapidly metabolized to other natural products. A minor metabolite, 1-ethyl-5,6-di-2,4(1H,3H)pyridinedione, was also identified and is postulated as an intermediate metabolite (EXTOXNET, 1997).This compound presents a relatively low acute toxicity, fact evidenced after tests with animal models (EXTOXNET, 1997). The chronic toxicity elicited by this compound is highly variable, since it depends on the species, the ranges of tested concentrations and the exposure period. Dogs administered with doses of 0, 50, 100 and 200 ppm of cymoxanil for 12 months did not show any evident signs of intoxications. However, rats treated for 90 days presented effects for doses of 1500 and 3000 ppm (No Observed Effect Level, NOEL, of 750 ppm), along with bodyweight and organ alterations, reduction in food consumption, testicular perturbations, and histopathological variations. Doses of 50, 500, 1750, 3500, 7000 ppm, for 90 days, were causative of severe toxicity in mouse, evident after 3 weeks. Females showed bodyweight and liver size reduction; exposed males had similar effects, but not as pronounced as observed in females (EXTOXNET, 1997). Experiments conducted with rats and rabbits did not show any evidence concerning teratogenic activity caused by cymoxanil (EXTOXNET, 1997). This compound showed potential mutagenic activity, since it induced chromosomal aberrations in Chinese hamster ovary (CHO) cells and in human lymphocytes (EXTOXNET, 1997). The reference dose for humans, defined by the United States Environmental Protection Agency (USEPA) that integrates NOEL values calculated for other species (namely rodents, concerning oncogenicity) is of 0.04 mg/kg/day (Cymoxanil NYS DEC Letter, 2003). Cymoxanil is toxic to aquatic organisms, such as fish species (*Cyprinius carpio* and *Lepomis macrochirus*), with lethal concentration 50 (LC_{50}) values of 91 mg/L (No Observed Effect Concentration, NOEC 47 mg/L) and 29 mg/L (NOEC 17 mg/L), respectively (EXTOXNET, 1997). Crustacean species are also susceptible to cymoxanil toxicity, as evidenced by the LC_{50} obtained for *Daphnia Magna*: 27 mg/L (NOEC of 15 mg/L) (EXTOXNET, 1997). Chronic ecotoxicity of this compound was also noticed, since exposure of *Daphnia magna* for 14 days allowed the calculation of values such as a NOEC of 0.067 mg/L, and a LOEC of 0.15 mg/L. In spite of the absence of indications that cymoxanil is environmentally persistent, its high partition coefficient can lead to moderate bioaccumulation by living organisms, including *P. clarkii* and humans. In humans, its bioaccumulation can result in neurotoxicity, as showed by Grandjean *et al*. (1999). However, interspecific differences may be important in the development of neurotoxicity. The potential neurotoxicity of cymoxanil was evaluated in rats as part of the 90-day feeding study at dietary levels of 0, 100, 750, 1,500 or 3,000 ppm. The NOEL for neurotoxicity was the highest dietary level tested, 3000 ppm for male (224 mg/kg/day) and female (333 mg/kg/day) rats. Cymoxanil is considered not to be a neurotoxicant (EXTOXNET, 1997). Nonetheless, the data reported in the Pesticide Fact Sheet (EPA, 1998) related cymoxanil exposure and myelin degeneration in the sciatic nerve axon of rodents, a finding that may partly explain the neurotoxic effects.

1.2. PROPINEB

Propineb is a dithiocarbamate, a class of compounds with high biological activity, being extensively used for agricultural purposes (Kazos *et al*., 2007). It is employed against plant diseases caused by fungal species (FAO, 2005). It is resistant to hydrolysis, but readily degraded by photoxidation (European Comission, 2003). It is rapidly metabolized, following oral intake, and may be excreted by urine or faeces (INCHEM, 1977). It is promptly absorbed, preferentially under its metabolite form (propionilthiourea). It is rapidly but uncompletely absorved (between 50 and 66 % of the administered dose). It can be metabolized following two distinct pathways, the formation of propilenothiourea (PTU), or the formation of propilenedia-

mine (PDA). It is excreted mainly in the form of propineb, and its excretion is complete after 48 hours (European Commission, 2003). It can be bioaccumulated, mainly in the thyroid and in the pituitary glands (INCHEM, 1977), causing potential neurotoxicity (Kazos *et al.*, 2007). This compound, as all dithiocarbamates, interferes with the synthesis and metabolism of proteins, due to its isocyanic metabolites. These intermediates cause the activation/inactivation of sulphidril groups (-SH) present in aminoacids, proteins and enzymes (Guven *et al.*, 1998). Its acute toxicity is generally low, since its lethal toxicity is above dosages of 5000 mg/kg for rats. Its chronic toxicity is highly variable, and depends upon the species and the time of exposure. The toxic effects on the thyroid gland were observed after exposure of mice to doses of 100 e 500 ppm, for 6 months. In a similar study, propineb showed again a tendency to induce morphological alteration in the thyroid gland of male rats, after an exposure to 10 ppm of propineb for a period of 63 days (INCHEM, 1993). Female rabbits treated with 250 mg/kg/day, 5 times a week for periods of 7 hours, for an overall exposure period of 3 weeks, via dermal administration, showed an increase in liver size (INCHEM, 1993). Dogs exposed for 2 years to 3000 ppm (75 mg/kg/day) of propineb did not show any evidence of morphological alterations. Female mice exposed for a period of 104 weeks to propineb, in a concentration of 800 ppm, showed an increased rate of ovary carcinogenesis (INCHEM, 1993). No evidence of increased rate of thyroid gland cancer was shown. Another long term (2 years) study involved the exposure of rats, and it showed increased mortality, body weight reduction, food intake decrease and muscular paralysis (INCHEM, 1993). *Post mortem* analysis showed increased weights of liver, kidney and thyroid gland, for the higher doses. Histopathology showed degeneration of the skeletal muscle (INCHEM, 1993). Propineb also alters the reproductive patterns of exposed animals. Rats, bred throughout 3 generations, and treated with doses of 20, 60, 200 and 600 ppm, exhibited lower generation times, lower weight at birth, and a lower number of offsprings. For the reproductive effects, this study found a No Observed Adverse Effect Level (NOAEL) of 60 ppm (3 mg/kg/day) (INCHEM, 1993). In the same study, propineb was also considered embryotoxic, since a dose of 100 mg/kg/day caused a depression in fetal growth and extremities dysplasia in animals born from mothers treated with this compound. In ecological terms, this compound is not toxic to terrestrial mammals nor to birds, since the LC_{50} calculated for these organisms was of 5000 mg/kg (Bayer CropScience, 2004). However, propineb is toxic to aquatic organisms, since a dosage of 0.4 mg/L is sufficient to kill half a population of rainbow trout (*Onchorhynchus mykiss*) (Bayer CropScience, 2004). As this compound is not hydrophilic, it can be bioaccumulated (at least in some extension) by humans, following chronic ingestion of contaminated organisms. In this case, it can exert toxic effects, at the thyroid gland, reproductive and carcinogenic levels.

1.3. **GLYPHOSATE**

It is a widely used herbicide, in formulations such as Roundup®, Rodeo®, and Accord®, (Tsui et al, 2005; Cox, 2000). This compound has proved to be highly successful due to its high persistency in soils (Araújo *et al.*, 2003). Glyphosate acts on plants due to the interference on the synthesis of aminoacids, nucleic acids and proteins (Coutinho and Mazo, 2005). Glyphosate is readily absorbed after oral intake, but only a small amount reaches the tissues. It is not accumulated, since it only accumulates in an extent of 1% after one week (European Commission, 2003). It is not extensively biotransformed, and is excreted in the unchanged form in 97% through urine and faeces (European Commission, 2003). The main metabolite is aminomethylphosphonic acid (AMPA) (INCHEM, 1994). This compound is toxic, particularly

at a chronic and sub-chronic level; acute toxicity by AMPA is negligible, since the calculated LD_{50} for rats was 8300 mg/kg of body weight. After sub-chronic exposure, AMPA causes increases in enzymatic activities (hepatic ethoxycoumarin O-deethylase and epoxide hydrolase), reduction of several organ weights and an excessive division of urinary epithelia (Cox, 2000; Hietanen et al, 1983). Glyphosate may be metabolized in the liver in small amounts, with consequent production of reactive oxygen species (ROS), major cause of its toxicity. This causes an overall modification of redox homeostasis, conducing to oxidative stress, which culminates in lipid peroxidation evidenced by increased formation of malonildialdehyde (MDA). Oxidative stress also causes damage on proteins, lipids and DNA, which may be responsible for the toxic effects caused by glyphosate (Beuret *et al.*, 2004). In spite of the above mentioned toxicity mechanisms, glyphosate is not acutely toxic, and its LD_{50} for rats is about 5600 mg/kg, and for rabbits, mice and goats it would be higher than 10000 mg/kg (EXTOXNET, 1997). Roundup® formulations have been associated to toxic phenomena, such as eye and skin irritation, increase of blood pressure, increase in heart rate, edema, nausea, palpitations, vomits, sore throat and fever (Cox, 2000). According to the same study, glyphosate may be toxic after long term administration of small amounts, since prolonged exposure of rats to dosages between 200 e 3400 mg/kg/day were causative of lesions in salivary glands. Long term studies, involving exposures of Charles River mice for 2 years, showed that glyphosate was able to cause significant modifications, such as body weight reduction, hepatic centrilobular necrosis, centrilobular hepatoycyte hypertrophy, and hyperplasia of urinary ephitelia (INCHEM, 1994). Occupational exposure to glyphosate has been related to increase rate of Hodgkins lymphoma (Cox, 2000). Other studies performed in rats for higher doses (30 mg/kg of body weight per day) showed an increase in testicular and pancreatic cancers (in males) and an increase in thyroid cancer in females (Cox, 2000; EPA, 1983; EPA, 1982). Glyphosate caused problems at the reproductive level, such as modification in the amount and quality of sperm, tumors in Leydig cells, reduction in production of sexual hormones, premature birth, spontaneous abortions (Cox, 2000), and also reduction of weight at birth (European Commission, 2003). Glyphosate exerts a negative effect on ecosystems, due to direct effects on exposed organisms, but also via modifications in the environment *per se*. Several insect species protect crops against pests, and their elimination may result in severe imbalances. The International Organization on Biological Control has demonstrated that the formulation Roundup® caused mortality of 80% of the populations of 4 species of beneficial insects (Cox, 2000). Glyphosate is only slightly toxic to aquatic organisms: calculated LC_{50} 96 h for several fish species are 120 mg/L (*Lepomis macrochirus*), 86 mg/mL (*Onchorhynchus mykiss)*, 168 mg/L (*Rasbora heteromorpha*; EXTOXNET, 1997). The use of formulations of glyphosate containing surfactants (e.g. Roundup®), increases the overall toxicity for the aquatic environment (EXTOXNET, 1997). Roundup® is approximately 30 fold more toxic than glyphosate alone. Glyphosate is moderately toxic to birds (LC_{50} = 2000 mg/kg body weight), but can affect them through the destruction of plants that are vital for their survival as food sources (Cox, 2000). A similar situation occurs for small mammalian species (Cox, 2000). Glyphosate is highly persistent in soils, and its half-life can vary between one week to 174 days. Microbial degradation of glyphosate can occur, leading to the formation of AMPA; this compound is extremely persistent in soils (half life of 240 to 958 days) (Watts and Macfarlane, 1999). In water, glyphosate is also persistent, with a half-life of 12 days to 10 weeks (EXTOXNET, 1993), due to its resistance to hydrolysis. As a consequence, glyphosate in the aquatic compartment is found bound to the sediments and to suspended matter (Watts and Macfarlane, 1999). Glyphosate may endanger human health, since it is expectable that several phenomena may derive from its consumption, such as mutations, cancer, and reproductive alterations. In terms of bioaccumulation, data published in the literature are not

conclusive about the possibility of glyphosate being accumulated by *P. clarkii*. However, the work by Watanabe *et al.* (2003) showed that the crustacean species *Procambarus acutis* (similar to *P. clarkii*) could accumulate several pesticide residues. Thus, it is not possible to exclude that *P. clarkii* may, at least to some extension, bioaccumulate glyphosate from the environment, and serve as a bioaccumulation vector for humans that ingest it as a food source.

2. BIOMARKERS USED FOR STUDYING PESTICIDE EFFECTS

Biomarkers are used for the early detection of biological responses consequent to the exposure of organisms to toxic compounds, in order to undertake protective measures. Biomarkers may substantiate modifications at the molecular, cellular, biochemical, and physiological levels, being determined in body fluids, tissues or organs, to indicate the exposure (Monteiro, 2003). According to Timbrell (1998), a biomarker is a tool for assessing the exposure to a toxic agent, and to evaluate its extension, in order to predict the most likely toxic response. According to this philosophy, the present conceptual approach involves the definition of potential biomarkers to be used for the toxicity assessment of pesticide exposure (mainly to cymoxanil, propineb and glyphosate) on the organism of *P. clarkii*. Several biomarkers that may be used for the environmental assessment of the toxic effects of pesticides have been already used for occupational studies, following human intoxications. The toxic potential of cymoxanil has not been profusely studied, and several studies that were already performed evaluate the toxicity of cymoxanil but always as a part of complex mixtures of pesticides. The study described by Bolognesi (2003) tried to assess the biological effects of pesticides (containing cymoxanil) on human workers, using the frequency of micronuclei from cells of the mouth mucosa as a biomarker. Results showed that the mixtures of pesticides did not elicit any modification of the selected parameter. However, a similar study by Lueero *et al.* (1999) was conducted to study the effects of pesticides (including cymoxanil) on human workers from greenhouses of the region of Almeria (Spain). This study revealed a slight increase in the micronuclei of mucosal cells of exposed workers, which may be partly due to the exposure to cymoxanil (14.1% of tested subjects used this compound). Carbonell *et al.* (1995) described a study for the assessment of effects of pesticides (including cymoxanil) on 29 workers from the region of Barcelona (Spain). The results showed a significant increase in the number of chromosomic aberrations, when comparing with the control population (not using pesticides). Furthermore, exposed workers also had higher activities of several plasmatic enzymes, such as cholinesterase, γ-gluthamiltransferase (GGT), oxaloacetic-gluthamic transaminase (SGOT), and pyruvic-gluthamic transaminase (SGPT). Several studies have already been published concerning the toxic effects of propineb, involving the assessment of biomarkers that may be used for the study of the contamination of several organisms by this pesticide. One of the studies, described by Guven *et al.* (1998) intended to assess the degree of accumulation and the histhological effects caused by the exposure to propineb and maneb on kidneys of female rats, and also on their fetuses. Results showed that, after 2 weeks of exposure to a concentration of 400 ppm, one could observe several modifications, both in mother and in fetuses, such as edema, degeneration of cells, severe hemorrhage, modifications and hyperplasia in renal tubules. We can thus conclude that histological damage may constitute a suitable biomarker for the assessment of damage induced by propineb in rats. Body weight may also function as a marker, since exposure for 2 weeks to concentrations of 200 and 400 ppm caused sensitive reduction in this parameter (Guven *et al.*, 1998). The generic effects caused by dithiocarbamate pesticides class (including propineb) have also been assessed in crustacean species. The study by Fingerman and co-workers (1998), shows

that limb regeneration may be severely inhibited in the shrimp species *Palaemonetes pugio* following exposure to dithiocarbamates. Human toxicological effects caused by exposure to propineb were assessed by Paz-y-Miño and co-workers (2004). This study showed evidences pointing to an involvement of pesticide exposure (including propineb) in an increased number of chromosomal and DNA aberrations in a human population in Ecuador. In spite of its indisputable importance, the study by Paz-y-Miño and co-workers (2004) has several confounding factors, such as the fact that the pesticide mixtures to which the human population is exposed are highly complex, and do not allow a straightforward resolution of effects due to individual substances.

Glyphosate causes several metabolic alterations in exposed organisms. The ingestion of glyphosate by pregnant female rats, at a concentration of 1% for a period of 21 days, caused modifications such as increased Thiobarbituric Acid Reactive Substances (TBARS) levels (indicating possible lipid peroxidation) and enhanced glutathione peroxidase activity (Beuret et al., 2004). The work by Hernández *et al.* (2006) showed that glyphosate was capable of *in vitro* inhibiting the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and acetilcholinesterase (AChE). However, *in vivo* results do not point to any modification caused by glyphosate. This conclusion was obtained after the work by Osten and co-workers (2004), who tried to establish a link between glyphosate chronic contamination and the possible effects on lactate dehydrogenase of the fish *Gambusia yucatana*. Other biomarkers that were already tested to assess the exposure of aquatic organisms to glyphosate were the activities of the enzymes Ca^{2+}-ATPase and cholinesterases, quantified in nerve glands of the mollusc *Phyllocaullis soleiformis* (Silva *et al.*, 2003), and the activity of glutathione S-transferases (GSTs) in the gills of the fish *Gambusia yucatana* (Osten *et al.*, 2004).

3. **CONCLUSIONS**

From the published literature, it was possible to conclude that *P. clarkii* may function as a contamination vector for pesticides to humans that use it as food source. Contamination of humans by pesticides may thus result in health problems, which may be more pronounced if the ingestion of contaminated organisms occurs for long time periods, even for vestigial amounts of pesticide residues. Among possible health effects, one can enumerate thyroid modifications caused by propineb, along with reproductive impairment and/or carcinogenesis. Cymoxanil, as a consequence of its lipophilicity, may easily cross biological membranes and exert neurological effects. Glyphosate is probably the less hazardous chemical, since it is not expectable to observe toxic responses after its consumption. However, if large amounts of this compound are ingested, which is not likely to occur in common situations, one cannot exclude the possibility of acute effects. For determination of the biological repercussions of pesticide intake, the present work presented potential methodologies that may be applied in environmental risk assessment. Effects caused by cymoxanil may be evaluated by the frequency of chromosomal aberrations, and through the quantification of the activities of enzymes, such as seric propionilcholinesterase (PChE), γ-glutamyltransferase (GGT), serum glutamic oxaloacetic transaminase (SGOT), and alanine aminotransferase (ALT). Limb regeneration may be used as a marker of exposure to propineb. Glyphosate effects may occur especially on oxidative stress homeostasis, and the levels of TBARS (indicative of the extension of lipid peroxidation) and glutathione peroxidase activity may be quantified in order to verify the exposure to this pesticide.

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