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Seasonal variation of *Sarpa salpa* fish toxicity, as related to phytoplankton consumption, accumulation of heavy metals, lipids peroxidation level in fish tissues and toxicity upon mice

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Abstract The aim of this work was to investigate for *Sarpa salpa* the seasonal trend in the food sources, heavy metals bioaccumulation and the oxidative stress in the organs. In addition, the toxicity was assessed by mouse bioassay of extract of the fish's organs collected in autumn, the peak of occurrence of hallucinatory syndrome. The toxicity was further studied for compounds present in epiphyte collected from the sea at the end of spring and in summer that are digested by the *S. salpa* in these seasons. We observed a higher lipid peroxydation in different tissues of *S. salpa* compared to the control fish *Diplodus annularis*. Furthermore, heavy metals accumulation in organs of these fish showed a significant variation between the two species ($P<0.05$). The lethal dose (LD50%) determined for crude ciguatoxin (neurotoxins) extracts of viscera, liver, brain and muscle of *S. salpa* were as follows: 1.217, 2.195, 14.395, 18.645 g/kg mouse, respectively. We noticed a significant correlation ($P<0.05$) between the total amount of toxic dinoflagellates and the level of TBARS in the liver, the brain and the muscle, this for all seasons and all sizes. Moreover, the cytotoxic effect observed for epiphytes extract confirms the transfer of toxins originating from toxic dinoflagellates, which live as epiphytes on *P. oceanica* leaves, to the fish by grazing. Our work indicates that, toxic phytoplanktons and heavy metals accumulation are responsible for the increase of oxidative stress in the organs of *S. salpa*. Hence, the edible part of *S. salpa*, especially the viscera and liver, can cause a threat to human health, and consumption should, for this reason, be dissuaded.

Keywords Diet · Heavy metals · Neurotoxins · Mouse bioassay · *S. salpa* · TBARS · Toxic dinoflagellate

Abbreviations

S. salpa	*Sarpa salpa*
D. annularis	*Diplodus annularis*
VI	vacuity index
TBARS	thiobarbituric acid-reactive substances
P. oceanica	*Posidonia oceanica*

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Introduction

Sarpa salpa, also known as salema porgy, is a species of bream which is an herbivorous sea fish that preferentially feeds on the seagrass *Posidonia oceanica* through the year (Peirano et al. 2001; Prado et al. 2008) and is used for human consumption in the Mediterranean region. Due to its low cost, this fish is predominantly on the menu of the lower income classes. The consumption of the *S. salpa* is, however, inadvisable in certain periods of the year because it causes a hallucinatory syndrome and central nervous system disorders. Most poisonings involving *S. salpa* consumption have been reported in spring and summer (Chevaldonne 1990; Raikhlin-Eisenkraft et al. 1989). An important observation in this context is the presence of ciguateric species that live as epiphytes on *P. oceanica* leaves (Ben Brahim et al. 2010) and are co-ingested by the *S. salpa* as part of their diet (Velimirov 1984).

The Gulf of Gabes is a threatened biotope mainly due to the pressure of anthropogenic expansion and dumping of large quantities of phosphogypsum and other chemical products which severely impacted benthic habitats (Hamza-Chaffai et al. 1999). It has been shown that epiphytes of seagrass are sensitive to environmental changes (Giovannetti et al. 2010). For example, various studies reported increases in epiphyte biomass parallel with nutrient enrichment (Armitage et al. 2006), eutrophication (Frankovich et al. 2009) and water quality (Meric et al. 2005). This has led to a substantial proliferation of microalgae and particularly of toxic dinoflagellates in the Gulf of Gabes (Turki et al. 2006). Such proliferation of undesirable microalgae has been shown to result in increasing problems in both coastal and estuarine environments (Smayda 1997; Leong and Taguchi 2005). For instance, ciguatera food poisoning increases due to the presence in fish of a toxin produced by the benthic alga *Gambierdiscus toxicus* and other coral microalgae, most of them belonging to these genera: *Prorocentrum*, *Ostreopsis* and *Amphidinum*. Another less common form of poisoning is ichthyoallyeinotoxism that is characterized by the development of central nervous system disturbances, especially hallucinations and nightmares (Halstead 1988; Chateau-Degat 2003). Several of the toxins increase to dangerous levels for humans during their transmission through herbivorous and carnivorous fish (Vaillant et al. 2001).

A second health threat associated with fish consumption comes from heavy metals; potentially all are harmful with interspecies differences in the tolerated level. Their presence in the environment has increased in some areas to levels which threaten the health of aquatic and terrestrial organisms, humans included. Therefore, numerous reports describe metal residues in wild fish from marine species (Sunlu et al. 2001; Romeo et al. 1999; Abou-Arab et al. 1996).

It is known for a wide range of environmental pollutants such as heavy metals and ciguatoxin (neurotoxins) that they can induce oxidative stress in aquatic animals including fish. The generation of reactive oxygen species (ROS) is commonly associated with cellular injures due to alterations in DNA, proteins and membranes (Leonard et al. 2004). Lipid peroxidation estimation has been found to have a high predictive value as a biomarker of this effect (Guilherme et al. 2008). Also, antioxidant enzymes have been proposed as biomarkers of contaminant-mediated oxidative stress in a variety of marine organisms, and their induction reflects a specific response to pollutants or toxins (Cossu et al. 1997). Because of, on one hand, the possible effects on the fish and, on the other hand, in relation to consumption of the fish by humans, it is relevant to study the oxidative stress in fish.

The objective of this work was to specify the seasonal trend of the food sources; heavy metals bioaccumulation and the oxidative stress in fish *S. salpa* organs. Moreover, toxicity was investigated by mouse bioassay of compounds present in the extract of fish organs collected in autumn, in epiphytes digested by *S. salpa* in summer and in the epiphytes collected from the Island of Kerkennah at the end of spring.

Materials and methods

Feeding behaviour and food composition of *S. salpa*

Specimen collection and preparation

The study was carried out off the Island of Kerkennah (Gulf of Gabes; Southeast Tunisia). This archipelago is characterized by extensive *P. oceanica* seagrass meadows (Hamza et al. 2000).

Specimens of *S. salpa* were collected between January 2006 and January 2007; 59 specimens in winter, 57 in spring, 57 in summer and 55 in autumn. The total lengths (TL) of the fish were measured to the nearest 0.1 cm and weight to the nearest 0.1 g. Their sizes ranged from 12.8 to 28 cm, and the fish were

divided into three classes according to Pallaoro et al. (2008): adults (3–7 years, large size) TL>20 cm; subadults (2 years, medium size) 17 cm<TL<20 cm and young (1 year, small size) TL<17 cm. Immediately after capture, the fish were dissected, the guts were removed and preserved in a 4 % formalin solution. Also, the liver, brain and muscles (including dark muscles) were removed, rinsed with ice-cold saline and stored at −80 °C until further analysis. In the laboratory, prey identification was carried out to the lowest possible taxonomic level. Species abundance and wet weight were recorded to the nearest ±0.001 g after removal of surface water by blotting paper.

As control, we collected the annular seabream *Diplodus annularis* (Linnaeus 1758) (belonging to the same biotope and the same family) between January 2006 and January 2007. Fifty specimens of *D. annularis* were collected in winter, 48 in spring, 51 in summer and 52 in autumn and were processed as described above.

Additional batches of *S. salpa* and *D. annularis* were collected in the same region for heavy metal assay, toxin extraction from stomach content and for mouse bioassay batches.

Analysis of the stomach contents

For each specimen collected whose stomach contained seagrass leaves and epiphytes, we determined the composition and food source. Therefore, the stomach contents were washed in a Petri dish and were studied under a microscope. Food items were sorted into large taxonomic groups and, when possible, identified to species level according to Fischer et al. (1987). The diet of the *S. salpa* was characterized using the vacuity index (VI) (1). Analysis of the vacuity index in time will inform us about the dietary behaviour of the fish and will allow us to formulate seasonal or monthly rhythms.

$$VI = \frac{Number\ of\ empty\ stomachs}{Number\ of\ investigated\ stomachs} \times 100 \qquad (1)$$

The second parameter is the relative mass of item *i* (2) where item *i* can be a group, a family, a genus or a species.

$$Relative\ mass(i)(\%) = \frac{Mass\ of\ the\ item(i)}{Total\ mass\ of\ stomachs\ contents} \times 100 \qquad (2)$$

Taxonomic identification and quantification of phytoplankton species in the stomach contents

The content of each stomach was put in a beaker, 500 ml of seawater was added and the mixture was shaken, filtered (75 μm pore size) and the flow-through preserved with 3 % formol.

Phytoplankton classification and counting was performed using an inverted microscope following the method proposed by Utermöhl (1958) after fixation with a Lugol's iodine solution (Bourrelly 1985).

The concentration of phytoplankton (non-toxic diatoms) and of toxic epiphytic dinoflagellates associated with ciguatera fish poisoning (e.g., *Prorocentrum* sp., *Ostreopsis* sp., *Coolia* sp., and *Amphidinum* sp.) was recorded.

Collection of epiphyte from marine ecosystems and toxin extraction

Epithytes were collected from four different locations in a *P. oceanica* meadow from depths of 5–15 m at the end of spring of 2006. *P. oceanica* leaves were put in a beaker, 250 ml of sea water was added, the mixture was shaken and filtered by vacuum filtration onto a Whatman GF/F glass fibre filter (diameter, 0.45 μm). Filters were immediately stored at −80 °C.

For toxin extraction, the filter was cut into three equal parts, each part was separately shaken for 3 h at 25 °C in the dark with 3 ml of one of the following solvents: ethanol, acetone or chloroform to extract the toxins. The solvent was collected and the extraction repeated with the same solvents; the combined extracts were subsequently dried at room temperature under flux (N_2). Finally, dried extracts were suspended in 2 ml 0.9 % saline+1 % Tween 60 and were sonicated for 5–10 min.

Collection of epiphyte from stomach contents of S. salpa and toxin extraction

For this experiment, 20 specimens of *S. salpa* were collected from different locations around the Island of Kerkennah during the summer of 2006. After the fish were dissected, their stomach content was put in a beaker, 250 ml of seawater was added, the content was shaken and filtered by vacuum filtration onto a Whatman GF/F glass fibre filter (diameter, 0.45 μm). Filters were then immediately stored at −80 °C.

The extraction of the toxins in the stomach contents of *S. salpa* was done using the same procedure as described for the toxins in epiphytes from marine environment.

Seasonal variation in cellular stress, heavy metals and marine toxins accumulated in tissue of the different organs of *S. salpa*

Biochemical assays in fish organs

The frozen organs (1 g), i.e., the liver, brain and muscle were homogenized (Ultra Turrax T25, Germany) in an ice-cold buffer (1/2, *w*/*v*; TBS, 50 mM Tris; 150 mM NaCl; pH 7.4) and centrifuged (5,000 g, 30 min, 4 °C), the supernatants were frozen at (−80 °C).

Lipid peroxidation was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of (Esterbauer 1993).

The protein content of tissue extracts was determined using the method of Lowry et al. (1951) using bovine serum albumin as reference standard.

Metal concentrations in fish organs

In this aspect, 15 *S. salpa* and 14 *D. annularis* were collected, their biometric data recorded (Table 1) and tissues used to determine the heavy metal content as described in materials and methods. Each organ sample (liver, viscera except liver and muscle including dark muscle) was treated according to the method described by Hamza-Chaffai et al. (1995). Lead (Pb), copper (Cu), and nickel (Ni) were analyzed on an atomic absorption spectrophotometry (HITACHI Z 8200) using the Zeeman Effect (Amiard et al. 1987). This methodology has been validated through international intercalibration exercises (Coquery and Horvat 1996).

Table 1 Biometric data (average±SE) of fish from the coastal waters of the Island of Kerkennah (Gulf of Gabes; South East Tunisia) during autumn 2006

Location	Species	Number	Length (cm)	Weight (g)
Island of Kerkennah	*Sarpa salpa*	15	21.9±0.66	113.86±1.50
	Diplodus annularis	14	20.14±0.44	105.93±1.50

Lipid-soluble extracts preparations from fish organs and LD_{50} determination

Samples for toxicity assay were prepared as follows: the fish muscle (including dark muscle) or organs (50–100 g) was thawed and cooked at 70 °C for 15 min in a water bag to denature proteins to enhance extraction efficiency during homogenization. Samples were cooled to room temperature, minced and extracted twice with acetone (3 L/kg flesh) using an explosion-proof homogenizer. The acetone filtrate was dried in a rotor evaporator at 55 °C water bath and 556 mbar vacuum, re-dissolved in 90 % of aqueous methanol (0.5 L/kg flesh) and extracted twice with hexane (1:1, *v*/*v*) to remove impurities from the aqueous methanol phase. The aqueous methanol portion was dried in rotor evaporator at 55 °C water bath and 337 mbar vacuum, re-dissolved again in 25 % of aqueous ethanol (0.5 L/kg flesh) and extracted with diethyl ether (1:1, *v*/*v*) three times. The extraction of lipophilic toxin from fish tissues was performed by subsequent liquid–liquid partitioning (separator funnel) as described by Lewis (2003). Diethyl ether extracts were concentrated by using rotor evaporator, re-dissolved in a known volume of chloroform–methanol (97:3, *v*/*v*) for quantification and were dried under nitrogen gas. The protein extracts were stored at −80 °C prior to testing.

In order to study the toxicity of marine toxin expected to be present in the samples, lipid-soluble extracts of the samples were analyzed using a mouse bioassay that was previously described by Vernoux (1994) and Lewis (1995, 2003). Mouse bioassay experiments were carried out using seven groups of male mice weighing 18–22 g (eight animals per group) purchased from the Central Pharmacy of Tunisia (SIPHAT, Tunisia). Animals were housed in a controlled environment (22±3 °C, 54–56 % humidity, a 12-h/12-h light–dark cycle). Mice were fed with a commercial balanced diet (SICO, Sfax, Tunisia) and drinking water was offered ad libitum. The body weight of the mice at the start of the experiment was measured. The ether-soluble extract was suspended in 1 % Tween 60/0.9 % saline at different concentrations, sonicated at 37 °C for 5–10 min, 0.8 ml (0.04 ml/1 g of mouse) was injected intraperitoneally (i.p.) and assayed in duplicate. Control mice were administered the same volume of 1 % Tween 60/0.9 % saline only. The mice were closely monitored at 1-h interval for 3–5 h after sample injection. Symptoms of intoxication

including hypothermia (rectal body temperature below 33 °C), diarrhoea, reduced locomotor activity and time of death of the mice (if this occurred within the first 24 h) were recorded. Symptoms or signs of intoxication in mice, other than the abovementioned, were rejected in this experiment to avoid subjective bias (Hoffman et al. 1983; Lewis 1995).

The diethyl ether extract containing marine toxin was quantified using the principle of the dose versus time-to-death relationship equation log (MU)=2.3 log (1+1/*T*), where MU is the number of mouse units (one MU=LD_{50} dose for a 20-g mouse) and *T* is survival time in hours of each mouse (Lewis and Sellin 1993; Lewis 1995, 2003).

Statistical analysis

Data are presented as average±standard deviation. The calculations were performed on groups of five animals each, and the differences were examined by a two-way analysis of variance (fixed factors: size and season), followed by the Fisher test (Stat View) and the significance was accepted at $^{*}P<0.05$. Also, correlation coefficients (*R*) were calculated for all sizes and all seasons together using the Pearson correlation.

Results

Feeding behaviour and food composition of S. salpa

Nature of the stomach contents of the S. salpa

Over the 1 year period over which this study was conducted (January 2006 to January 2007), we found that the diet of large- and medium-sized *S. salpa* was composed of the following varieties of marine flora: marine Phanerogams (seagrass): *P. oceanica*; red algae: *Petiola stupulaceae*; *Grateloupia* sp.; *Ceramium* sp.; *Sphacelaria pluma*; brown algae: *Dictyota dichotoma*; *Cytoseira* sp. and the Cyanophyta: *Lyngbia* sp. In spite of the abundance of the *Caulerpa prolifera* in the biotope, this species did not appear in the stomach contents.

Vacuity index (VI)

To study the dietary behaviour of *S. salpa*, we examined 228 stomachs, 104 of which contained macrophytes species. The *S. salpa* were captured at night when the fish are generally most active because they are feeding. The individuals caught were hauled onboard the following morning, therefore some may have stayed in the net for several hours, and their capture may have occurred before the ingestion of prey or after digestion. As a result, many specimens had an empty stomach at the moment they were collected.

The seasonal variation of the mean vacuity index for the medium and large size classes showed a decrease in spring (31.57 %) and summer (40 %) relative to winter (78.9 %) or autumn (66.10 %), indicating that *S. salpa* fed significantly more in the first two periods compared to winter (Table 2). The mean annular VI for *S. salpa* was 54.38 %.

Diet of the S. salpa

P. oceanica is the year around the major constituent of the diet of large- and medium-sized *S. salpa* (yearly average, >50 %). However, we observed a significant variation between the seasons. A striking difference was found in winter; the large *S. salpa* generally fed less on *P. oceanica* leaves which comprised 7.34 % of their diet with a large contribution of red algae (87.18 %) and brown algae (5.77 %) (Fig. 1). Unlike the large, the medium-sized fish almost exclusively fed on *P. oceanica* leaves in winter 97.34 % (Fig. 1).

During spring, the fish tend to eat more as demonstrated by a lower vacuity index (Table 1). During spring, both classes of fish nourish on a diverse diet of seagrass, brown algae, red algae and for the medium-sized fish also on *Cyanophyta Lyngbia* sp. The high contribution of *Cyanophyta Lyngbia* sp. in their diet (up to 40 %) is certainly not because of preference but rather because of the abundance of these algae in the environment (Fig. 1).

Summer is the season preceding the reproductive period for large-sized *S. salpa*. Large fish increased their grazing of *P. oceanica* leaves, reaching 79.5 % during this season, whereas the medium incorporated also a significant percentage of *Lyngbia* sp. (42.18 %) into their diet (Fig. 1).

Table 2 Seasonal variation of the vacuity index for the large- and medium-sized *S. salpa* throughout the year of study

Season	Winter	Spring	Summer	Autumn
Vacuity index (%)	78.94	31.57	40.00	66.10

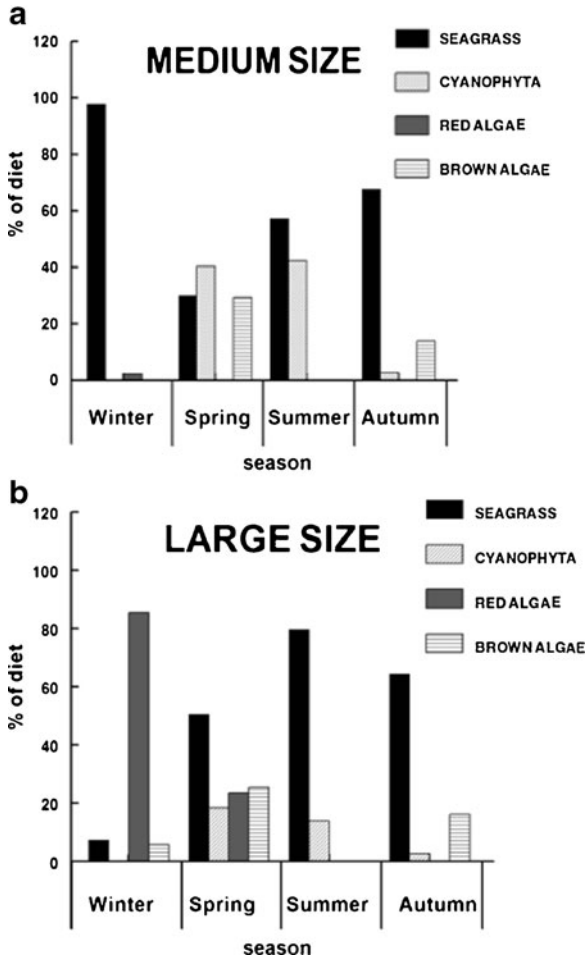

Fig 1 Grazing behaviour of *S. salpa*. Diet composition was determined in the stomach content for two size classes; medium (2 years) (TL<20 cm) (**a**) and large (3–7 years) (TL>20) (**b**) in terms of percentage of seasonal grazing (percent)

Both medium and large size classes attained maximum grazing *P. oceanica* leaves (64.31 %; 67.20 %, respectively) during autumn (Fig. 1). We noticed that its food spectrum in this period was much diversified with a multitude of algae. So was the brown alga *Cytoseira* sp. only in this season identified with a percentage of 4.24 %.

Evaluation of the phytoplankton composition

The toxic phytoplankton species observed in the stomach contents of *S. salpa* were the dinoflagellates *Prorocentrum mexicanum*, *Prorocentrum lima*, *Prorocentrum concavum*, *Ostreopsis siamensis*, *Coolia monotis* and *Amphidinum carterae*. Seasonal variation of the toxic phytoplankton was compared to the total number of phytoplankton in the *P. oceanica* meadow. We found that the proportions of the toxic species followed the same pattern as found for the total phytoplankton population (Fig. 2).

Seasonal variation in cellular stress, heavy metals and marine toxins accumulated in tissue of the different organs of *S. salpa*

Seasonal variation of lipid peroxidation for the S. salpa

The levels of lipid peroxidation (TBARS) in different organs and compartments of the *S. salpa* specimens were studied. The results were compared with those found for the control fish, the annular seabream *D. annularis*. The organs were selected on the basis of functional criteria, which made them preferential targets, i.e., xenobiotic metabolism (liver) and the known neurotoxic effect of toxic dinoflagellates ingested by *S. salpa* (brain) and muscle being the preferred part of the fish used for human consumption.

We noted several differences: in winter, no significant difference was found for the TBARS in the medium and large size classes of *S. salpa* compared to *D. annularis* of the same size class (Fig. 3a).

In spring, there was only a significant increase found of the TBARS in the liver of the large-sized *S. salpa* to its control (Fig. 3b).

In summer, increased levels of TBARS were found in the livers of both large- and medium-sized *S. salpa*.

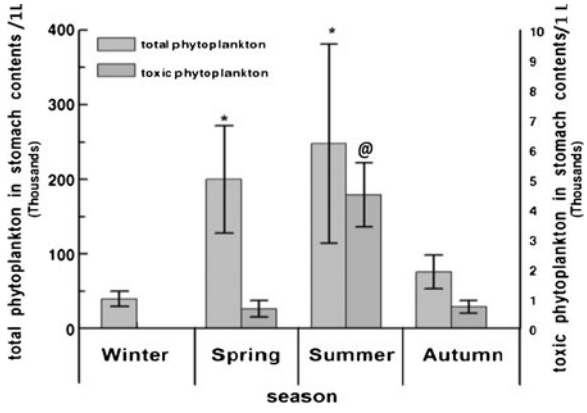

Fig. 2 Seasonal variation of the toxic phytoplankton. The total number of phytoplankton counted in 1 L of water from the stomach contents of *S. salpa* was analyzed. *$P<0.05$: for total phytoplankton compared to autumn or winter; $^{@}P<0.05$: for toxic phytoplankton compared to autumn, winter or spring

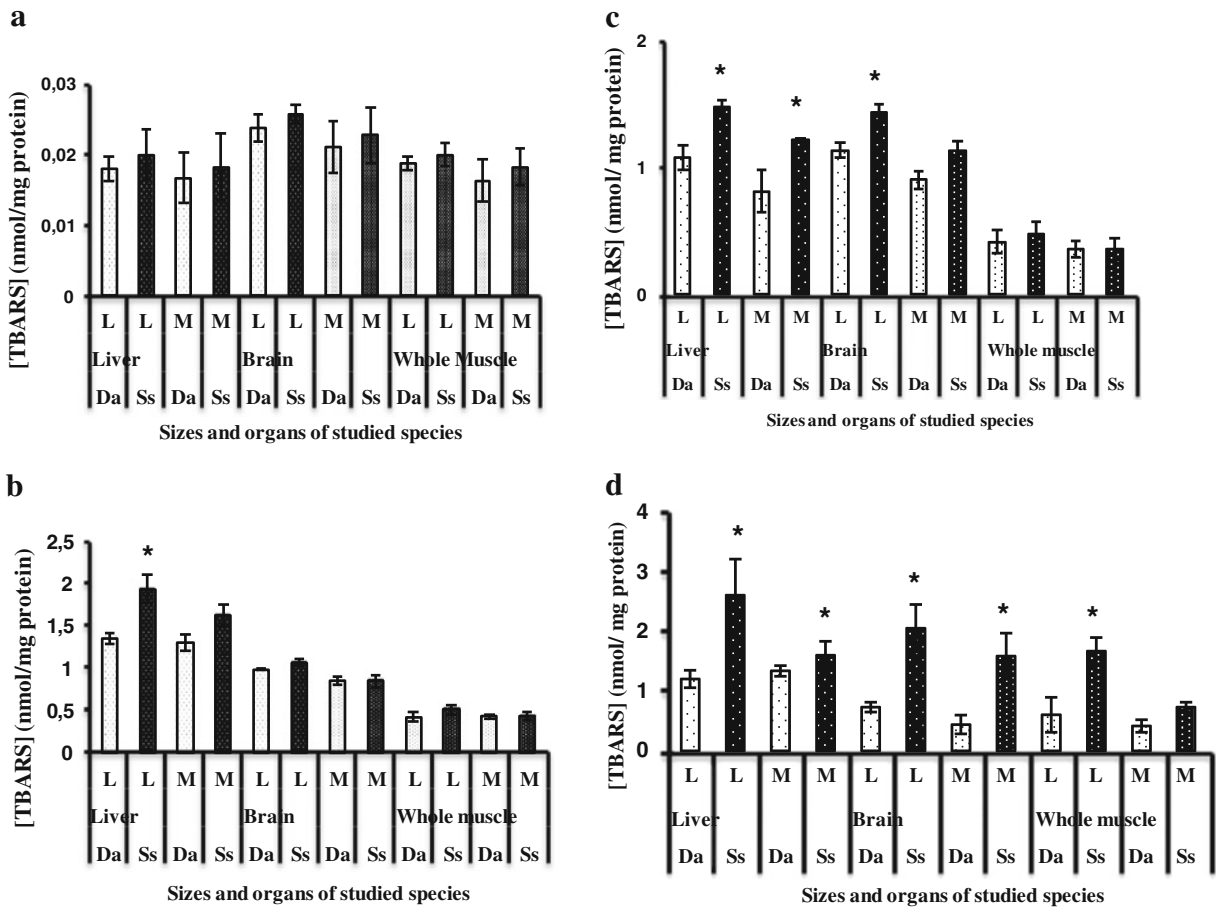

Fig. 3 The level of TBARS in the liver, brain and muscle (including dark muscle) during winter (**a**), spring (**b**), summer (**c**) and autumn (**d**) for the *S. salpa* compared to the control fish *D. annularis* for the large (3–7 years) (TL>20) and medium (2 years) (TL<20 cm) size classes. The values represent the average of 5 measurements±SD. *$P \leq 0.05$ indicates significant difference between *S. salpa* (*Ss*) and control fish, *D. annularis* (*Da*)

In addition, we observed a significant increase of the TBARS in the brain for the large size class (Fig. 3c).

In autumn, we observed significantly higher levels of lipid peroxidation in the liver, brain and muscle (including dark muscle) of large size class *S. salpa* and in the liver and brain of medium size class *S. salpa* compared to their size matched *D. annularis* controls (Fig. 3d).

Concentration of heavy metals in different organs

The mean concentrations of lead, copper and nickel in the liver, muscle (including dark muscle) and viscera (viscera except liver) of *S. salpa* and *D. annularis* are shown in Table 3. We observed a significant increased concentration of all three heavy metals in each of the three tissues; viscera, liver and muscle of *S. salpa* investigated compared to the control fish *D. annularis*.

LD_{50} determination of marine toxin accumulated in the organs of S. salpa

After extraction with diethyl ether, we obtained the crude ciguatoxins (neurotoxins) from 50 g of fish sample: viscera (viscera except liver) 1.16±0.20 g (mean±SE); liver 1.02±0.15 g (mean±SE); brain 0.5 ±0.11 g (mean±SE); muscle (including dark muscle) 0.69±0.20 g (mean±SE).

For the LD_{50} determination, we used six experimental groups and one control group, each with eight mice. Affected mice exhibited typical signs of neurotoxicity disorders including hypothermia (rectal body temperature <33 °C; trembling), a significantly reduced locomotor activity during the first 2 h and failure breathing and no evident signs of gastrointestinal problems (e.g. diarrhoea). Results are given in Table 4.

Table 3 Mean values (microgrammes per gram dry weight) with standard deviations of copper, nickel and lead in various body organs of two fish species *S. salpa* and *D. annularis* collected from the Island of Kerkennah (Gulf of Gabes; South East Tunisia) during autumn 2006

Fish species	Heavy metals	Liver	Muscle	Viscera
Diplodus annularis	Cu	2.46±0.28	1.11±0.01	2.00±0.04
	Ni	0	0	0.62±0.06
	Pb	1.12±0.09	0	0.38±0.19
Sarpa salpa	Cu	9.54±0.50*	1.86±0.11*	7.48±0.78*
	Ni	1.01±0.11*	1.22±0.04*	2.25±0.15*
	Pb	1.70±0.18*	0	1.40±0.18*

Values represent the average±SE; $n=8$

* $P \leq 0.05$ significant from control

The difference between toxin concentrations of the liver, viscera and brain extracts was significant as compared to muscle extract, also a significant difference ($P<0.05$) between the liver and viscera organ since concentrations of toxins in organs are defined in ascending order: muscle, brain, liver and viscera.

Additionally, after extraction of epiphyte from the aquatic environment (different stations from the Island of Kerkennah) and stomach contents of *S. salpa*, we obtained the crude microalgae toxins from 250 ml epiphyte water, respectively: 300±2.88 mg; 295±4.08 mg (mean±SE).

The difference between toxin concentrations of epiphyte extracts from *P. oceanica* and stomach content of *S. salpa* confirms the transfer of toxins originating from toxic epiphytic phytoplankton that live on the *P. oceanica* leaves to the fish organs by grazing (Table 5).

Inter-seasonal correlation between the total toxic algae dinoflagellates and the markers of oxidative stress in different organs of S. salpa

The level of lipid peroxidation (TBARS) in *S. salpa* shows a cumulative effect over the consecutive seasons, beginning in spring with a maximum effect during autumn and affecting an increasing number of organs, first the liver, then the brain, and finally the muscle. This effect further increases with the size of the animal, which might be related to the amount of *P. oceanica* leaves consumed that are enriched with toxic epiphytic phytoplankton in a similar seasonal pattern.

To justify and consolidate our precedent observations, we started a series of tests of find a correlation between the total toxic dinoflagellates in the stomach contents of *S. salpa* and the profiles of oxidative stress in fish organs. Pearson correlation analysis is listed in Table 6. There were significant positive correlations between the total toxic dinoflagellates and the level of TBARS measured in the liver, the brain and the muscle for all seasons and all sizes together.

Discussion

Feeding behaviour of *S. salpa*

Over the year, we see a fluctuating pattern in the vacuity index for *S. salpa*. This can probably be

Table 4 Concentration of toxicity expressed in mouse units per 100 g tissue and microgrammes per 100 g tissue, estimated in fish organs of *S. salpa* collected from the Island of Kerkennah (Gulf of Gabes; South East Tunisia) during autumn (2006)

	Concentration of toxicity (MU/100 g tissue)	Concentration of toxicity (μg/100 g tissue)	DL50% g/kg of mouse
Liver extract	24±0.40	481.75±94.46	2.195
Viscera extract	48.25±0.85	792±147.8	1.217
Brain extract	2.87±0.025	65.75±7.4	14.395
Muscle extract	0.75±0.004	13.62±1.13	18.645

Values are average±SE; $n=8$

Table 5 Toxicity concentration of epiphytes extract from *P. oceanica* collected during spring (2006) and epiphytes extract of stomach contents collected from the Island of Kerkennah (Gulf of Gabes; South East Tunisia) during summer (2006) expressed respectively in mouse units per 100 ml of epiphyte water and in mouse units per stomach contents

	Concentration of toxicity (MU/100 ml of epiphyte water; MU/stomach content)
Epiphyte extract from *P. oceanica*	0.46±0.0025 MU/100 ml of epiphyte water
Epiphyte extract from stomach contents	1.05±0.0062 MU/stomach contents

Values are average±SE; n=8

explained by the feeding behaviour of *S. salpa*. Grazing in June–September is done in massive schools that actively feed on *P. oceanica* to accumulate reserve for the winter period when they eat less and to prepare adult fish for reproduction. *S. salpa* has a single period of maximum spawning, from mid-September to mid-October followed by a period of intensive settlement at the end of November. In March, when adults migrate to deep waters, juveniles live and feed in shallow, rocky bottoms (Peirano et al. 2001). This results in a mean annular VI for *S. salpa* that is rather weak (54.38 %) in contrast to that found for *D. annularis* (91.48 %) (Derbal et al. 2007). Our findings are consistent with this behaviour, before egg-laying the *S. salpa* nourishes and stores lipids for the sake of their genital product maturation.

The grazing behaviour for the medium and large size classes of *S. salpa* is in essence only different in winter. The medium sized in winter almost exclusively feed on seagrass, whereas the large sized feed on red algae. In spring, both classes have a mixed diet of with near equal contribution of seagrass, cyanophyta and brown algae (and in the case of large sized, also red algae). In summer and autumn, the contribution of seagrass increases. These results are comparable to the results found by Alcoverro et al. (1995). Not withstanding regression during the last decades, the seagrass meadow is in equilibrium with the environment and *P. oceanica* shows a regular cycle in both plant growth and succession of epiphyte colonization. Adult leaves, which showed the greatest colonization by epiphytes, were preferred by herbivores throughout the year at all depths (Tomas et al. 2005; Young et al. 2005). The variation of the toxic phytoplankton in stomach contents of *S. salpa* follows a seasonal trend with a peak in summer.

Seasonal variation in cellular stress in the *S. salpa* as a result of fluctuations in their diet

Oxidative stress is related to the formation of ROS, which are continuously generated endogenously during normal metabolism and as byproducts of biotransformation reactions of toxins or xenobiotics. Cellular antioxidant status is used to evaluate the ability of organisms to resist an environmental stress such as those induced by some marine pollutants (Frenzilli et al. 2004).

Looking at the feeding behaviour of *S. salpa*, it would seem that the low TBARS levels in winter are due to the absence of toxic epiphytic dinoflagellate on *P. oceanica* leaves consumed. In spring, the increase in

Table 6 Correlation matrix (Pearson test) between the total toxic algae dinoflagellates in stomach contents of *S. salpa* and the level of TBARS for all seasons and all sizes together in fish organs

	Total toxic dinoflagellate in stomach contents of *S. salpa*	Level of TBARS in liver	Level of TBARS in brain	Level of TBARS in muscle
Total toxic dinoflagellate in stomach contents of *S. salpa*	1			
Level of TBARS in liver	0.263*	1		
Level of TBARS in brain	0.646**	0.990**	1	
Level of TBARS in muscle	0.734**	0.975**	0.990**	1

* P<0.05; ** P<0.01 numbers of parameters 2 and number of analysed samples 48

the percentage of *P. oceanica* as the food source for the large-sized fish could explain the difference found between the TBARS level in the livers of *S. salpa* vs. *D. annularis*. Moreover, we noticed that an increase in the TBARS level is certainly due to the presence of toxic algae dinoflagellate in the stomach contents of *S. salpa* with a percentage equal to 1.14 %. It should be noted that many other factors can contribute to the formation of the TBARS, particularly the age of the organisms and the type of food (Bocquené and Galgani 1998). In summer, *P. oceanica* leaves showed greatest colonization by epiphyte owing to the fact that the surrounding water was calmer. *S. salpa* had maximum periods of grazing on *P. oceanica* leaves during summer and autumn. This has an effect on the TBARS level in the livers and also in the brain of both large- and medium-sized *S. salpa*. The brain is very susceptible to oxidative damage due to the high content in cellular membranes containing unsaturated lipids and to the high rate of oxygen metabolism (Giuffrida-Stella and Lajtha 1987). However, the brain is not particularly enriched in antioxidant enzymes (Benzi and Moretti 1995). In autumn, fish from both size classes consumed *P. oceanica* leaves as the preferential food source (>50 %). The percentage of the ciguateric species, compared to the other seasons, was the highest (5.26 %) and this reflected the rate of TBARS level with significantly higher levels for *S. salpa* in all tissues investigated. These observations enabled us to conclude that in autumn there was a peak in toxicity (oxidative stress) in *S. salpa* and that the toxicity is now even present in the muscle of the large-sized fish.

Accumulation of heavy metals: additional source of cellular stress with species differences

S. salpa and *D. annularis* have very different eating habits which take place in different trophic chains. *D. annularis* is an omnivorous fish that feeds on worms, mollusks, crustaceans algae, *P. oceanica* leaves and as secondary prey annelids (Bradai 2000). *S. salpa* is an herbivorous sea fish that nourishes preferentially on the seagrass *P. oceanica* throughout the year (Tomas et al. 2005; Prado et al. 2008). Several studies suggest employing seagrasses as bio-indicators of coastal water metal contamination (Ferrat et al. 2003). *P. oceanica* may have a greater bioaccumulation capacity for all the metals considered except Hg and may reflect both contaminations in the water column and in sediment (Lafabrie et al. 2007). This could explain the quantities of heavy metals bioconcentrated significantly in organs of *S. salpa* as compared to the control fish, *D. annularis*. The accumulation of heavy metals in organs of *S. salpa* might contribute to the increase of oxidative stress in our study. The evaluation of lipid peroxidation, which results from the oxidative injury of saturated and unsaturated lipids, has been broadly used as a marker of the induction of oxidative damage in fish suffering from metal-induced environmental stress (Ahmad et al. 2006; Gioda et al. 2007).

We compared our average values of copper in fish organs (1.86–9.54 $\mu g\ g^{-1}$) with the Canadian food standards (Cu, 100 $\mu g\ g^{-1}$), Hungarian standards (Cu, 60 $\mu g\ g^{-1}$) and the range of international standards (Cu, 10–100 $\mu g\ g^{-1}$) this showed that our values are lower than the guideline. The lead values in fish organs were found to be in the range of 1.40–1.70 $\mu g\ g^{-1}$. These values were lower than those reported in the range of international standards for Pb in fish is 0.5–10 $\mu g\ g^{-1}$. Nickel values in fish organs were found to be in the range of 1.01–2.25 $\mu g\ g^{-1}$. Nickel contents in the literature have been reported in the range of 0.11–12.88 μg/g dry weight in fish species from Iskenderun Bay, Northern East Mediterranean Sea, Turkey (Turkmen et al. 2005), 0.02–3.97 μg/g in seafoods from Marmara, Aegean and the Mediterranean Sea in Turkey (Turkmen et al. 2008).

Methodology used

Mouse bioassay (AOAC 1980; Lewis 2003) remains the accepted regulatory method for detection and quantification of many marine toxins in suspect samples, so as to protect the health of the public. The assay may also quantify lethal and sublethal doses of marine toxins that are found in coral fish extracts (Louzao et al. 2004).

These toxins (especially neurotoxins, e.g. ciguatoxins, brevotoxin) structure cannot be destroyed through cooking, refrigeration and weak acid treatments (Bruslé 1997; Guzmán-Pérez and Park 2000). It is also well-known that even the application of temperature up to 120 °C would not reduce the toxicity of ciguateric fish (Pottier et al. 2002).

In our study, standard laboratory criteria include hypothermia (rectal body temperature below <33 °C, trembling), symptoms of intoxication (reduced locomotors activity, respiratory failure). These are objective parameters to determine the presence of toxin in

fish organs also both epiphyte from stomach content and *P. oceanica*, especially for those in sublethal amounts.

Three clinical reports were published about possible ciguatera poisoning in humans after consumption of fish caught in the eastern Mediterranean. One case involved the *S. salpa* (Spanier et al. 1989; Bentur and Spanier 2007), two other reports, one recently published, involved rabbitfishes, *Siganus* sp. (Herzberg 1973; Raikhlin-Eisenkraft and Bentur 2002). Within a few hours, specific signs of poisoning occur including delirium, visual and/or auditory hallucinations (often involving animals), depression and feelings of impending death with reactive tachycardia and hyperventilation and disturbed behaviour. If they are able to sleep, patients classically report terrifying nightmares (De Haro et al. 2003).

The interaction between herbivores and seagrass can be mediated by epiphytes (Tomas et al. 2005; Young et al. 2005), at least in part, because seagrasses do not appear to be an attractive food source (Bulleri et al. 1999; Hereu 2006) as the presence of phenolics in them proves to be a source of chemical deterrents (McMillan 1984). Ciguatoxins are produced by *Gambierdiscus toxicus* epiphytic dinoflagellates living on macroalgae and other substrates in tropical areas. Other dinoflagellates have also been suspected (e.g. *Amphidinium carterae*, *Coolia monotis*, *Prorocentrum lima*, *P. concavum*, *P. rhathymum* and *Ostreopsis siamensis* (Swift and Swift 1993).

Therefore, we noticed that the toxicity in the muscle and brain of *S. salpa* were lower than the toxicity in the viscera and liver. Shellfish exhibiting any detectable level of toxicity by mouse bioassay are considered potentially unsafe for human consumption. In practice, a value of 20 MU/100 g (USFDA 2005) is considered the guidance level at or above which shellfish are prohibited from harvest. Moreover, we noticed that the cytotoxic compounds present in different organs of this fish can pose a threat to human health and is a source of intoxication especially in the visceral part. This study demonstrated the effectiveness of the mouse bioassay to determine the edibility of the studied fish.

Inter-seasonal correlation between the total toxic algae dinoflagellates and the marker of lipid peroxidation for the *S. salpa*

The induction of the increase in lipid peroxidation response was a logical answer to the exposure to toxic substances generated by toxic dinoflagellates and heavy metals accumulation. *S. salpa* and *D. annularis* have very different eating habits, take place in different trophic chain. This explains the quantities of heavy metals bioconcentrated significantly in organs of the *S. salpa* as compared to the control fish, *D. annularis*. The accumulation of heavy metals in organs of the *S. salpa* might be implicated in the increase of oxidative stress in our study. So, the accumulation of heavy metals in organs of *S. salpa* may intervene, but phytoplankton is most influential because of the toxic dinoflagellates consumed by *S. salpa*. The control fish, *D. annularis*, does not consume toxic dinoflagellates and does not show an oxidative stress like *S. salpa*. It appears that oxidative stress is generally increased in the presence of toxic phytoplankton in the stomach contents of *S. salpa*.

Algal toxins represent a major global hazard to public health. Ensuring seafood contains safe concentrations of these toxins, many of which can induce toxic effects such as neurotoxicity, is one of the major challenges to the shellfish/aquaculture industries as well as to regulatory authorities. The metabolism of toxic compounds frequently results in the formation of ROS, which significantly contribute to their toxicity (Chovanec et al. 2003). As stated above, heavy metals can be ruled out as important contributors to oxidative stress in relation to the hallucinatory syndrome and central nervous system disorders. A positive correlation was observed between the total toxic dinoflagellates and the TBARS level at the side of the liver, the brain and the muscle for all seasons and all sizes together.

Conclusions

We noticed a significant correlation between the total toxic dinoflagellates in the stomach contents and the TBARS level at the side of the liver, the brain and the muscle for all seasons and all sizes together. In addition, we found that the toxicity in fish organs correlates with the rate of dietary intake containing *P. oceanica* leaves which are rich in toxic epiphytic phytoplankton of a seasonal nature. The cytotoxic effect of epiphytes extract confirms the transfer of toxins originating from toxic epiphytic phytoplankton that live on the *P. oceanica* leaves to the fish organs by grazing. Furthermore, the significant accumulation of heavy metals in organs of *S. salpa* might contribute to the increase in oxidative stress found in our study.

Our work strongly indicates that toxic dinoflagellates and heavy metal accumulation are responsible for the increase of oxidative stress level in fish organs of the *S. salpa*. Hence, the liver and viscera of this fish can cause a threat to human health, and consumption should, for this reason, be dissuaded.

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