

THE BIOLOGY OF *OREOCHROMIS NILOTICUS*
IN A POLLUTED CANAL

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Abstract

In the present investigation, some biological aspects (length-weight relationship, condition factor, age, growth, mortality, gonadosomatic index and fecundity) of *Oreochromis niloticus* population from Shanawan drainage canal, Al-Minufiya Province, Egypt, were studied. This was carried out on 162 fish samples collected during the period from April 1992 to May 1993. *Oreochromis niloticus* was found to mature earlier (8-9 cm), more fecund (18126 eggs/year) and has high mortality rates. A comparison of the various parameters of *Oreochromis niloticus* from Shanawan drainage canal with those of other authors in different localities and times in Egypt revealed year-to-year and geographical significant differences in relation to variation in weight with length, condition, fecundity, growth and mortality rates. These differences were discussed and found to be attributed to the effect of eutrophication and pollution on the growth, age and other biological aspects of *Oreochromis niloticus*. However, multiple regression analysis showed that only female gonadosomatic index correlated significantly with pesticides and heavy metals.

Introduction

Over the last years, in many African countries a considerable population growth has taken place, accompanied by a steep increase in urbanization, industrial and agricultural land use. This has entailed a tremendous increase in discharge of a wide diversity of pollutants to receiving water bodies and has caused undesirable effects on the different components of the aquatic environment and

on fisheries (FAO, 1992 and Authman, 1998). Furthermore, modern agricultural activities have introduced several polluting substances such as organic matter, chemical fertilizers, insecticides, herbicides, etc., into the River Nile and drainage systems.

Of the 5.5 billion cubic meters (bcm) of water released from the Aswan High Dam, about 50 percent ends up in the drainage system where Egypt possesses approximately 50000 Km of irrigation and drainage canals (Redding & Midlen, 1990). Drainage water above the Delta is returned to the Nile River, recycled downstream and reused.

Drainage water, however, consists not only of irrigation return water but in many cases industrial and domestic wastewater where huge volumes of untreated wastewater are discharged into agricultural drains daily. Drainage water is therefore contaminated with salts, agricultural chemicals (Pesticides & Heavy metals) and other pollutants as pathogens from domestic sewage and industrial discharge.

In spite of the intensive studies conducted on the biology of *Oreochromis niloticus* in River Nile and Egyptian lakes (e.g. Al-Zahaby *et al.*, 1981; Khallaf *et al.*, 1986 and Ezzat *et al.*, 1990), it was found that the biological studies on this species in drainage canals were neglected and our knowledge about this is deficient. Therefore, for the first time, this work is introduced to study some biological aspects of *Oreochromis niloticus* in Shanawan drainage canal located in the Delta region, Al-Minufiya Province, Egypt. In this canal, pollution by various concentrations of pesticides and heavy metals in water as well as in *Oreochromis niloticus* were identified earlier by Khallaf *et al.* (1994, 1995 and 1998).

Material And Methods

Study Area

Shanawan drainage canal extends about 8 km from Manshat Shanawan village through Almay village near Shebeen Al-Koom city, into another big drainage canal in Menouf city, called Sabal drainage canal which is connected to it at Shoubrapass village and which finally drains into the Rossetta Branch of the River Nile.

Shanawan drainage canal is very shallow, where its depth ranges between 1 - 1.5 m, and narrow, where its width in the first 1.5 Km of its length ranges between 2.5 - 3.5 m, while the width of the remaining part averages up to 6.5 m.

Oreochromis niloticus specimens were caught by bottom trap (Gobiah) nets by fishermen during the night between 5 pm and 6 am, at different localities in Shanawan drainage canal within a 5 km length, during consecutive months between April 1992 to May 1993. The present study is based on a total of 162 *Oreochromis niloticus*; 87 males and 75 females. Measurements of fish scales, length, weight and gonad were carried out in the laboratory after collection of the fish.

Statistical analyses were carried out at the 5 % level of significance. Statistical analysis followed those of Sokal & Rohlf (1981) and Dixon & Massey (1983). Statistical tests of the difference between regression coefficients and multiple regressions were carried out using STATGRAPH (Ver. 5) computer program.

Results and Discussion

(1) The length - weight relationship :

In this study, the values of the weight-length exponent are : $n = 2.65052$, 2.70562 and 2.70308 for male, female and combined sexes, respectively; of the standard length-weight relationships of *Oreochromis niloticus* in Shanawan drainage canal. However, comparing the weights of the different lengths of both sexes shows that the female is generally heavier or more robust than male. On a seasonal basis, *Oreochromis niloticus* from Shanawan drainage canal attained its highest average weight in winter (86.9 gm), autumn (75.6 gm) and spring (72.6 gm), while the lowest one was observed in summer (58.0 gm).

As shown in Table 1, there was a significant difference ($F = 10.08$ & $P < 0.05$) between the regression coefficients (n) of the length-weight relationship of the fish.

The weight at total length of *Oreochromis niloticus*, in Shanawan drainage canal, is heavier when compared to those from other Egyptian localities, with the exception of fishes of length groups 11, 16 and 19 cm compared with those of Bahr Shebeen Canal 1986 and length groups 21 and 23 cm compared with those of Lake Nasser 1994 (Table 2). The difference in weight becomes wider

with increase in length, reaching its maximum of 56.7 gm at 17 cm length. The higher weight at some length intervals of fishes of Bahr Shebeen than those of Shanawan drainage canal may be explained by the running water effect, low salinity, low nitrogenous load and favourable content of dissolved oxygen of the fluvial Bahr Shebeen (Authman, 1990; Elewa & Authman, 1991), i.e. clear water (free from pollution). However, food availability is high in Shanawan drainage canal due to the high content of phytoplankton, water plants and zooplankton and the high amounts of organic matter. Also, higher values of pesticides and heavy metals in water and different organs of the fish of Shanawan drainage canal (Khallaf *et al.*, 1994, 1995 & 1998) cause enlargement of these organs leading to increase in their weight.

However, the lower value of condition factor of *O. niloticus* in Shanawan drainage canal compared to the values of the same species in Bahr Shebeen, Lake Manzalah and Lake Nasser means that there is a general stress on the fish population of that species in this canal. In accordance, in earlier studies, similar results were also attributed to the high levels of pollution (Sindermann, 1979 & 1990; Lowe-McConnell, 1975 & 1987).

The comparison of growth in length was carried out and shown in table (3). It was found that the maximum growth in total length of *Oreochromis niloticus* from Shanawan drainage canal occurred during the first year of life (50.3 %), followed by a decrease in the second year (49.7 %). These results indicated that, the growth in length of fishes of Shanawan drainage canal was higher than those of other waters with the exception of the fishes of age-group I from the River Nile.

However, growth increment in weight at the end of the first year is very small (14.9 %), then it sharply increased during the second year of life (85.1 %). Again, these differences may be attributed to the size of fish and ecological conditions (as pollution) of different localities. It was mentioned by many authors (Hosny, 1987; Akel, 1989; Bakhoum & Faltas, 1994) that the growth of fish decreased in the polluted water. Akel (1989) and Bakhoum & Faltas (1994) reported that *O. aureus* (a very closely related species) in a non polluted region of Lake Mariut grew faster and had better growth performance index than those of a polluted region of the lake.

(2) *Survival and mortality rates :*

The annual survival rates of male and female *O. niloticus* from Shanawan drainage canal were 0.09 and 0.06, respectively. This indicates that females suffer higher mortality than males. However, these values are lower than those of *Oreochromis niloticus* from other Egyptian localities (Table 4). Equally, the annual mortality rate (0.92) and instantaneous mortality rate (2.56) of the studied fish were higher than those of other localities. This may be attributed to the highly increased eutrophication and pollution of the water of Shanawan drainage canal. Saleh (1980) and Dethlefsen & Tiews (1985) mentioned that pollution increased the susceptibility of fish to diseases and increased the mortality rates. Hasan & Thomas (1993) mentioned that the acute pollution condition of the Lake Mariut proper water has now come to be the main factor causing the remarkable decrease in catch.

(3) Gonadosomatic Index (GSI) and fecundity :

In comparison to other localities, fecundity ranged between 1234 to about 3893 eggs for fishes ranging in standard length from 9 cm (= 12 cm TL) to 18 cm (= 23 cm TL) as compared to 482 to 3982 eggs for the fish ranging in total length from 11 to 22 cm in Bahr Shebeen Canal (Alne-na-ei, 1986), 290 to 924 eggs of the fish ranging in total length from 13 to 18 cm in the middle region of Lake Manzalah (Shalloof, 1991), 453 to 1383 eggs of the fish ranging in total length from 9 to 22 cm in Lake Mariut (El-Shazly, 1993) and 547 to 3670 eggs of the fish ranging in total length from 12 to 27 cm in the River Nile (Tharwat, 1995). However, the higher fecundity of *O. niloticus* in Shanawan drainage canal as compared to the same species in other localities is due to the response to drastic conditions concerning various pollutants recorded in the studied area (Khallaf *et al.*, 1994, 1995 & 1998; Authman, 1998). In accordance, Bagenal (1960 a & b), Nikolsky (1963) and Lagler *et al.* (1977) stated that enormous fecundity in fishes is related to enormous mortality. They also reported that an increase in fecundity of an individual within the population represents an adaptive response of the population to environmental changes where an increase in the fecundity ensures the preservation, and not the extermination, of the species; it ensures its relative stability both in space and in time, in the event of fairly wide fluctuations in the environmental conditions. The hypothesis that fecundity showed increase with increasing environmental harshness could be fully proved in this study. Thus, GSI and fecundity in this study correlated significantly with pollutants such as nitrates, phosphates, silicates and organic matter. In addition to those, GSI correlated positively with heavy metals and pesticides (Table 5).

Oreochromis niloticus fish in Shanawan drainage canal mature earlier (8 & 9 cm), were more fecund (18126 eggs/year) and have an extended spawning season (September-May) and high mortality rates. In accordance, Balon (1979 & 1981), Noakes & Balon (1982), and James & Bruton (1992) stated that increasing environmental harshness leads to earlier sexual maturity at a smaller size, extended spawning season, increased fecundity and high mortality.

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Table (1) : Comparison of the regression coefficient (n) of the total length-weight relationship of *Oreochromis niloticus* from Shanawan Drainage Canal (present study) with those of *Oreochromis niloticus* from different localities : Bahr Shebeen Canal (Alne-na-ei, 1986), Lake Manzalah (Shalloof, 1991), Lake Mariut (El-Shazly, 1993; Bakhoum, 1994), Lake Nasser (Mekkawy *et al.*, 1994) and River Nile (Tharwat, 1995).

	Source of variation	Sum of squares	df	Mean squares	F ratio
Shanawan Drainage Canal X Bahr Shebeen Canal	Among n's	0.000046	1	0.000046	0.4613
	Both regressions	0.0043	31	0.0001	
Shanawan Drainage Canal X Lake Manzalah	Among n's	0.0005	1	0.0005	0.5464
	Both regressions	0.0241	26	0.0009	
Shanawan Drainage Canal X Lake Mariut (1993)	Among n's	0.0212	1	0.0212	107.31*
	Both regressions	0.0074	32	0.0002	
Shanawan Drainage Canal X Lake Mariut (1994) [Southeast basin]	Among n's	0.0042	1	0.0042	20.69*
	Both regressions	0.0040	22	0.0002	
Shanawan Drainage Canal X Lake Mariut (1994) [Lake proper]	Among n's	0.0104	1	0.0104	34.59*
	Both regressions	0.0064	22	0.0003	
Shanawan Drainage Canal X Lake Nasser	Among n's	0.0158	1	0.0108	5.44*
	Both regressions	0.1461	51	0.0029	
Shanawan Drainage Canal X River Nile	Among n's	0.0143	1	0.0143	141.80*
	Both regressions	0.0028	29	0.0001	

* Significant Difference ($\alpha = 0.95$).

Table (3) : Comparison of calculated total lengths (cm) and weights (gm) of *Oreochromis niloticus* at different localities : Shanawan Drainage Canal (present study), Bahr Shebeen Canal (Alne-na-ei, 1986), Lake Manzalah (Shalloof, 1991), Lake Mariut (El-Shazly, 1993), Lake Nasser (Mekkawy *et al.*, 1994) and River Nile (Tharwat, 1995).

Locality		Age-groups					
		I	II	III	IV	V	VI
Shanawan Drainage Canal	T.L.	9.1	18.1				
	Incr.	9.1	9.0				
	% incr.	50.3	49.7				
Bahr Shebeen Canal	W	19.4	130.3				
	Incr.	19.4	110.9				
	% incr.	14.9	85.1				
Lake Manzalah	T.L.	7.1	12.5	17.0	19.7		
	Incr.	7.1	5.4	4.5	2.7		
	% incr.	36.1	27.4	22.8	13.7		
Lake Manzalah	W	9.6	46.6	109.9	165.7		
	Incr.	9.6	37.0	63.3	55.8		
	% incr.	5.8	22.3	38.2	33.7		
Lake Manzalah	T.L.	6.8	10.0	13.0	17.6		
	Incr.	6.8	3.2	3.0	4.6		
	% incr.	38.6	18.2	17.1	26.1		
Lake Manzalah	W	8.4	23.1	46.6	107.2		
	Incr.	8.4	14.7	23.5	60.6		
	% incr.	7.8	13.7	21.9	56.6		

Table 3 Continued

		I	II	III	IV	V	VI
Lake Mariut	T.L.	8.4	14.2	18.8	24.2		
	Incr.	8.4	5.8	4.6	5.4		
	% incr.	34.7	24.0	19.0	22.3		
W	W	9.6	48.4	116.5	256.6		
	Incr.	9.6	38.8	68.1	140.1		
	% incr.	3.8	15.1	26.5	54.6		
Lake Nasser	T.L.	14.9	19.4	23.7	29.4	33.5	37.8
	Incr.	14.9	4.5	4.3	5.7	4.1	4.3
	% incr.	39.4	11.9	11.4	15.1	10.8	11.4
W	W	71.5	153.6	277.6	517.8	759.1	1036.8
	Incr.	71.5	82.1	124.0	240.2	241.3	277.7
	% incr.	6.9	7.9	12.0	23.2	23.3	26.7
River Nile	T.L.	13.0	19.1	22.6	25.1		
	Incr.	13.0	6.1	3.5	2.5		
	% incr.	51.8	24.3	13.9	10.0		
W	W	41.7	138.7	233.1	320.5		
	Incr.	41.7	97.0	94.4	87.4		
	% incr.	13.0	30.3	29.4	27.3		

T.L. = total length (cm).
W = weight (gm).
Incr. = increment.

Table (4) : Comparison of the survival and mortality rates of *Oreochromis niloticus* from Shanawan Drainage Canal (present study) and those from different localities.

Locality	Author	Annual Survival rate (S)	Annual Mortality rate (A)	Instantaneous Mortality Rate (Z)
Lake Tiberias	El-Bolock & Koura (1961)	0.24	0.76	1.42
Lake Mariut	El-Zarka <i>et al.</i> (1970) El-Shazly (1993)	0.24 0.17	0.76 0.73	1.44 1.78
Lake Manzalah	Bishara (1973) Shalloof (1991)	0.38 0.45	0.62 0.55	0.97 0.80
Jebel-Aulia (Sudan)	Mahdi <i>et al.</i> (1973)	0.08	0.92	2.49
Wadi El-Rayan	Soliman (1981)	0.76	0.24	0.27
Lake Nasser	Azim (1974) (1965 – 1970) Latif & Khallaf (1984) (1987) (1985) Mekkawy <i>et al.</i> (1994)	0.31 0.35 0.31 0.11	0.69 0.65 0.69 0.89	1.18 1.06 1.16 2.25
Bahr Shebeen	Alne-na-ei (1986) Latif <i>et al.</i> (1989) Khallaf 1992)	0.25 0.25 0.22	0.75 0.75 0.78	1.40 1.40 1.52
River Nile	Tharwat (1995)	0.33	0.67	1.12
Shanawan Drainage Canal	Present study	0.08	0.92	2.56

Table 2. Comparison of average observed weight (gm) at different total lengths (cm) of *Oreochromis niloticus* from different localities : Shanawan Drainage Canal (present study), Bahr Shebeen Canal (Alne-na-ei, 1986), Lake Manzalah (Shalloof, 1991), Lake Mariut (El-Shazly, 1993; Bakhoum, 1994), Lake Nasser (Mekkawy *et al.*, 1994) and River Nile (Tharwat, 1995).

Total length(cm)	Shanawan Drainage Canal	Bahr Shebeen Canal			Lake Manzalah (middle region)			Lake Mariut 1993		
		Weight	W	D	% D	W	D	% D	W	D
9	19.7	—	—	—	15.1	+ 4.6	23.35	11.7	+ 8.0	40.61
10	24.8	24.1	+ 0.7	2.82	22.4	+ 2.4	9.68	17.8	+ 7.0	28.23
11	33.4	34.1	- 0.7	2.10	31.6	+ 1.8	5.39	22.0	+ 11.4	34.13
12	40.3	42.5	- 2.2	5.46	38.2	+ 2.1	5.21	30.2	+ 10.1	25.06
13	51.1	53.1	- 2.0	3.91	46.2	+ 4.9	9.59	36.8	+ 14.3	27.98
14	63.8	64.5	- 0.7	1.10	52.5	+ 11.3	17.71	46.0	+ 17.8	27.90
15	75.5	78.1	- 2.6	3.44	71.5	+ 4.0	5.30	59.7	+ 17.6	23.31
16	91.5	93.5	- 2.0	2.19	80.0	+ 11.5	12.57	70.6	+ 20.9	22.84
17	110.2	108.6	+ 1.6	1.45	91.8	+ 18.4	16.70	86.5	+ 23.7	21.51
18	130.6	125.0	+ 5.6	4.29	118.0	+ 12.6	9.65	98.8	+ 31.8	24.35
19	145.3	147.2	- 1.9	1.31	138.0	+ 7.3	5.02	117.6	+ 27.7	19.06
20	179.0	168.5	+ 10.5	5.87	—	—	—	140.9	+ 38.1	21.28
21	194.6	194.0	+ 0.6	0.31	155.0	+ 39.6	20.35	161.0	+ 33.6	17.27
22	—	220.2	—	—	—	—	—	179.2	—	—
23	257.5	255.0	+ 2.5	0.97	250.0	+ 7.5	2.91	221.9	+ 35.6	13.38

W = Weight (gm).

D = difference.

% D = % difference.

Table 2 Continued

Total length(cm)	Lake Mariut 1994 Southeast basin (non-polluted)			Lake Mariut 1994 Lake Proper (polluted)			Lake Nasser			River Nile (Cairo sector)		
	W	D	% D	W	D	% D	W	D	% D	W	D	% D
9	12.6	+ 7.1	36.04	11.3	+ 8.4	42.64	—	—	—	16.4	+ 3.3	16.75
10	16.9	+ 7.9	31.85	13.0	+ 11.8	47.58	20.0	+ 4.8	19.35	21.3	+ 3.5	14.11
11	22.1	+ 11.3	33.83	20.7	+ 12.7	38.02	—	—	—	27.7	+ 5.7	17.07
12	27.7	+ 12.6	31.27	26.8	+ 13.5	33.40	—	—	—	36.6	+ 3.7	9.18
13	35.9	+ 15.2	29.75	34.2	+ 16.9	33.07	36.7	+ 14.4	28.18	46.1	+ 5.0	9.78
14	44.5	+ 19.3	30.25	43.6	+ 20.2	31.66	46.7	+ 17.1	26.80	56.4	+ 7.4	11.60
15	52.0	+ 23.5	31.13	54.6	+ 20.9	27.68	51.9	+ 23.6	31.26	71.0	+ 4.5	5.96
16	70.2	+ 21.3	23.28	64.6	+ 26.9	29.40	57.5	+ 34.0	37.16	85.1	+ 6.4	6.99
17	83.5	+ 26.7	24.23	77.5	+ 32.7	29.67	53.5	+ 56.7	51.45	97.7	+ 12.5	11.34
18	96.7	+ 33.9	25.96	93.0	+ 37.6	28.79	75.0	+ 55.6	42.57	121.7	+ 8.9	6.81
19	117.0	+ 28.3	19.48	108.0	+ 37.3	25.67	—	—	—	141.8	+ 3.5	2.41
20	133.7	+ 45.3	25.31	128.0	+ 51.0	28.49	167.5	+ 11.5	6.42	175.1	+ 3.9	2.18
21	—	—	—	—	—	—	202.5	- 7.9	4.06	192.9	+ 1.7	0.87
22	—	—	—	—	—	—	227.5	—	—	220.4	—	—
23	—	—	—	—	—	—	284.4	- 26.9	10.45	253.2	+ 4.3	1.67

W = Weight (gm).

D = difference.

% D = % difference.

Table (5) : Significant relationships of some biological parameters of *Oreochromis niloticus* from Shanawan Drainage Canal against some physico-chemical parameters, heavy metals and pesticides via the multiple regression analysis.

Interaction		a	b ₁	b ₂	DF	r ²	F	P
TLc (cm)	VS	19.6436	- 0.0488		2 , 1	0.9951		0.0692
SLc (cm)	Water temperature (°C) and Photoperiod (hrs) *	14.7720		- 0.1622	2 , 1		49.7483	0.0984
Wtc (gm)	VS Water temperature (°C) and Photoperiod (hrs) * Ammonia (mg/L) and Nitrite (mg/L) *	169.5619	- 0.7092	- 5.3729 - 1.5340	2 , 1	0.9413	8.0108 50.7404	0.0975
GSI _m	Organic matter (mg/L) and Nitrate (mg/L) *	- 0.9298		- 0.0063	2 , 1		13.0788	0.1893
GSI _m	VS Organic matter (mg/L) and Phosphate (mg/L) *		0.0291	- 0.0008		0.9820	27.2415	
GSI _m	VS	- 0.9095	0.0277		2 , 1	0.9648		0.1850
GSI _m	Nitrate (mg/L) and Phosphate (mg/L) *	- 0.1353		- 0.0029	2 , 1		1368.81	0.0189
	VS Heavy metals (mg/kg) and Pesticides (mg/kg) in	6.4625	- 0.0003		2 , 1	0.9679		0.1767
Fec.	Organic matter (mg/L) and Nitrate (mg/L) *	- 5993.66		- 43.5197	2 , 1		14.1849	0.1820
Fec.	VS Organic matter (mg/L) and Phosphate (mg/L) *		185.997	- 5.5758		0.9852	33.3099	
Fec.	VS	- 5852.70	176.719		2 , 1	0.9677		0.1774
Fec.	Nitrate (mg/L) and Phosphate (mg/L) *	- 805.962		- 19.0648	2 , 1		436.687	0.0334

$$Y = a + b_1 X_1 + b_2 X_2$$

where Y
X is the independent variable.
is the intercept.
b
r²
D
F
F F
P
hypothesis.

* =
TLc =
= standard length of combined sexes.
Wtc =
weight of combined sexes.
GSI_m =
GSI_f =
Fec. =

**A COMPARATIVE TOXICOLOGICAL STUDY
OF THE PIKE (*Esox lucius* L.) FROM TWO LOCALITIES
IN THE RIVER DANUBE
WITH DIFFERENT LEVELS OF POLLUTION**

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Abstract

A comparative toxicological research of the pike (*Esox lucius* L.) from the River Danube was performed during a one-year cycle, at two localities with a different level and type of pollution. Comparative analyses of the enzyme activity (aspartate aminotransferase - AST, alanine aminotransferase - ALT, alkaline phosphatase - ALP), and concentrations of the total protein, urea and creatinine in the pike sera at these two localities, show that the occasional significant changes (Mann-Whitney U-test) in these parameters occur due to the sublethal pollution. Differences between the localities were also manifested as changes in the value of the hepatosomatic index (HSI). Under the influence of pollution, changes in the diet were present as well, determined by examining the stomach contents. These changes were accompanied by the significant changes in the slopes of the length-weight regressions ($\log W = a + b \log TL$). All changes were reversible.

Introduction

The pike (*Esox lucius* L.) represents a species with a wide circumpolar range in the Northern Hemisphere, having a commercial and ecological value both in North America and in Europe and Asia (Scott and Crossman, 1973). This species is a top predator in aquatic ecosystems. It generally represents a stationary species and is the predator of a sedentary type (Gonczi et al., 1985). All these figures, as well as the fact that many experiments were conducted on this species, both in laboratory conditions and in natural populations, make it very suitable for pollution monitoring in aquatic ecosystems (Johnson and Bergman, 1984; Balk, 1985). The aim of this research was to determine the importance of the biochemical parameters of the pike blood sera, hepatosomatic index, diet, and length-weight relationship as indicators of the population condition at two localities exposed to a different type and level of pollution.

Material and Methods

The pike was sampled monthly with an electrofishing appliance (230 V, 16.5 A) at two localities during one year. The upstream locality (km 1175) has a group of islets and channels that represent a typical pike habitat, while the downstream locality (km 1163) represents a true river course. There are no greater pollution spills at the upstream locality, except during the period of the low water level, when water from the channels and the flooded area carries a large amount of organic pollution. The downstream locality is constantly exposed to pollution from the largest city collector sewer and from the wastewater of a paper mill.

A total of 569 specimens of pike were sampled, of which 352 were from the upstream and 217 from the downstream locality. The analysed specimens were sexually mature (determined through histological cross-sections of gonads). The total body length (TL, cm) and the total body weight (W, g) were measured in all specimens.

The sampling of blood and biochemical analyses (aspartate aminotransferase - AST, alanine aminotransferase - ALT, alkaline phosphatase - ALP, total protein, urea, creatinine), were done according to the procedure described in one of the previous papers by the author (Lenhardt, 1992).

The presence or absence of the food in the stomach was determined in all specimens.

The length-weight relationship was estimated for each month and for each locality using the linear regression of logarithmically transformed data for length and weight of the body:

$$\log W = \log a + b \log TL$$

The comparison of the values of biochemical parameters and the HSI was done with a test of rank sums (Mann-Whitney U-test). The significance of differences in percentage of the present dietary items was estimated with a Yates corrected χ^2 test. The comparison of the regression slopes was done with an analysis of variance.

Results

The comparative analysis of the biochemical parameters of the pike sera in specimens sampled at two localities indicates that there is a relative increase of certain parameters during the year (Table 1).

In February, at the upstream locality, water from the flooded area and the channels, loaded with a great amount of organic matter, entered into the main stream due to the low water level. During that time, a significantly higher activity of the transaminases (AST, ALT) and the increased concentration of urea were observed in specimens sampled at the upstream locality in comparison with the downstream locality (Mann-Whitney U-test). The analysis of the diet indicates that there are significant differences in the percentage of the full stomachs at two analysed localities during January ($\chi^2 = 9.02$, d.f.=1, $P < 0.01$) and February ($\chi^2 = 4.72$, d.f.=1, $P < 0.05$), with lower values detected at the upstream locality (Table 1). The comparative analyses show that there are significant differences between the regression slopes [$F_{(1,60)} = 7.655$, $P < 0.01$] of the length-weight relationship in pike specimens sampled during February at two analysed localities, with lower values of the coefficient b at the upstream locality, which indicates poor dietary conditions at this locality.

During June, the activity of the transaminases was increased at the downstream locality, while the concentration of urea was increased both during June and July. The data show that there was a significantly less number of full stomachs (Table 1) during July ($\chi^2 = 9.31$, d.f.=1, $P < 0.01$) at the downstream locality, as well as that the difference between the regression slopes of the length-weight

relationship during June [$F_{(1,27)} = 6.900$, $P < 0.05$], July [$F_{(1,53)} = 6.616$, $P < 0.05$] and August [$F_{(1,52)} = 5.780$, $P < 0.05$] was significant, with lower values of the coefficient b at the downstream locality.

The increased values of biochemical parameters during May at the upstream locality were probably caused by the time interval between sampling at the downstream and the upstream locality (15 days). During this time the water temperature increased from 16°C to 20°C, which probably influenced the increase of the concentration of creatinine, which is correlated with water temperature (Lenhardt, 1992), while the increased values of ALT can be explained with the correlation of this parameter with the photoperiod (Lenhardt, 1997).

Urea had significantly higher values at the downstream locality four times during the year, in the period of low water level, while in November the activity of transaminases and the concentration of creatinine were increased too, which points to a higher frequency of the sublethal pollution at this locality.

The increased values of creatinine in males, in March and April, at the downstream locality, were probably related to spawning activities, because the individuals with groups of unabsorbed spermatozoa (cross-sections of gonads) were found until mid June at the downstream locality (Lenhardt, 1997).

Occasionally there was a relative increase of the total proteins in males during March and June at the downstream locality, whilst at the upstream locality this increase occurred both in males and females during September.

The relative increase of the HSI during certain periods of the year was probably related to the reproductive cycle.

Table 1. Comparative analysis of investigated parameters at upstream (UL) and downstream locality (DL), F-female, M-male, * P<0.05, ** P<0.01.

month			Dec	Jan	Feb	Mar	Apr	May
AST (U ⁻¹)	F	UL	310.4	411.0	286.6	583.8	314.9	481.6*
		DL	301.6	374.0	221.3	441.5	424.0	275.4
AST (U ⁻¹)	M	UL	357.4	386.1	302.6*	445.0	345.9	421.4
		DL	290.8	395.8	264.8	451.1	388.8	385.2
ALT (U ⁻¹)	F	UL	10.6	11.2	7.4	5.7	5.3	11.0*
		DL	10.6	10.7	4.4	5.9	5.6	6.6
ALT (U ⁻¹)	M	UL	7.0	8.8	6.4*	5.8	5.2	10.5
		DL	7.4	8.6	3.6	5.5	4.7	7.5
ALP (U ⁻¹)	F	UL	50.9	54.2	44.2	91.8	56.8	59.4
		DL	47.1	67.1	58.6	59.5	47.1	53.6
ALP (U ⁻¹)	M	UL	45.9	39.5	39.5	55.2	46.4	63.4
		DL	45.2	42.7	38.8	48.9	54.8	57.7
total protein (g ^l ⁻¹)	F	UL	36.0	30.7	31.0	28.2	34.8	33.3
		DL	41.2	34.6	31.1	37.6	33.7	36.4
total protein (g ^l ⁻¹)	M	UL	39.0	36.3	37.4	27.7	36.2	34.8
		DL	36.9	36.0	40.0	33.5*	41.7	33.8
urea (mmol ⁻¹)	F	UL	0.63	0.57	0.62	0.83	1.16	1.18
		DL	1.18	0.94*	0.58	1.00	0.77	1.22
urea (mmol ⁻¹)	M	UL	0.85	0.68	0.68*	1.06	1.17	1.07
		DL	1.07	0.91*	0.52	0.93	1.15	0.98
creatinine (μmol ⁻¹)	F	UL	0.28	0.28	0.24	0.26	0.49	0.72*
		DL	0.26	0.28	0.32	0.36	0.44	0.57
creatinine (μmol ⁻¹)	M	UL	0.48	0.46	0.42	0.39	0.54	0.83*
		DL	0.49	0.46	0.48	0.52*	0.66*	0.55
HSI	F	UL	3.61	4.16*	2.66	1.57	1.32	1.28
		DL	3.26	3.22	3.38	1.86	1.50	1.53
HSI	M	UL	2.20	1.87	2.06	1.40	1.38	1.34
		DL	1.81	1.87	2.26	1.48	1.26	1.44
regression slope - b		UL	3.22	3.08	3.07	2.91	3.10	3.37
		DL	3.15	3.16	3.23**	3.19	3.10	3.38
full stomachs (%)		UL	52.0	10.0	7.4	37.5	28.6	26.7
		DL	60.0	58.3**	33.3*	57.1	44.4	44.4

Table 1. (continued)

month			Jun	Jul	Aug	Sep	Oct	Nov
AST (U ⁻¹)	F	UL	252.0	313.5	307.8	321.5	301.3	306.7
		DL	386.0	436.7	323.6	325.3	331.0	442.4*
AST (U ⁻¹)	M	UL	274.1	415.0	308.8	313.8	342.8	335.0
		DL	439.0*	365.5	328.8	304.0	308.8	451.3*
ALT (U ⁻¹)	F	UL	6.1	5.9	8.4	5.0	5.1	7.4
		DL	9.6	7.2	7.8	5.6	5.6	8.8
ALT (U ⁻¹)	M	UL	7.8	5.9	8.0	4.9	5.2	7.4
		DL	11.4*	6.6	7.9	5.1	5.6	8.3*
ALP (U ⁻¹)	F	UL	50.3	57.4	62.9	87.5	58.4	49.4
		DL	66.2	53.8	57.6	62.5	46.3	63.7
ALP (U ⁻¹)	M	UL	60.9	52.3	64.0	54.0	57.1	56.0
		DL	64.9	58.3	61.3	60.4	66.2	69.6
total protein (g ^l - ¹)	F	UL	31.6	36.6	36.1	40.1*	34.5	32.9
		DL	36.0	38.0	34.2	29.6	33.8	36.8
total protein (g ^l - ¹)	M	UL	34.3	37.8	38.1	37.5*	33.8	38.7
		DL	39.6*	40.5	36.8	32.8	35.2	39.5
urea (mmol ⁻¹)	F	UL	1.12	0.99	1.28	1.46	1.34	1.03
		DL	1.20	1.48*	1.40	1.58	1.35	1.40*
urea (mmol ⁻¹)	M	UL	1.11	1.12	1.45	1.52	1.38	0.99
		DL	1.50*	1.84*	1.44	1.59	1.38	1.43*
creatinine (μmol ⁻¹)	F	UL	0.84	1.13	1.34	0.56	0.37	0.31
		DL	0.66	1.19	0.92	0.62	0.31	0.32
creatinine (μmol ⁻¹)	M	UL	0.94	1.24	1.20	0.84	0.47	0.42
		DL	0.82	1.17	1.16	0.74	0.54	0.52*
HSI	F	UL	1.28	1.72	1.71	1.49*	2.02	2.61
		DL	1.24	1.35	1.28	1.06	1.94	2.27
HSI	M	UL	1.48	1.61	1.57*	1.45*	1.40	1.84*
		DL	1.46	1.54	1.27	1.04	1.16	1.34
regression slope - b		UL	3.06*	3.11*	3.11*	3.08	3.27	3.16
		DL	2.66	2.90	2.97	3.06	3.21	3.11
full stomachs (%)		UL	54.5	76.9**	25.0	25.7	32.4	51.9
		DL	33.3	31.6	20.0	36.4	16.7	37.9

Discussion

The presented results point to the importance of biochemical parameters as "early warning indicators" of water pollution (Hodson, 1986), but also to the complexity of such an approach to pollution monitoring. Field-sampled fish generally show greater variability and wider range of values of blood parameters in comparison with the laboratory-reared fish (Edsall, 1999). Therefore, to use these parameters for diagnostic purposes, it would be necessary to acquire an estimate of their standard values (Lockhart and Metner, 1984; Folmar, 1993), as well as their correlation to exogenous and endogenous factors (Larsson, 1985). The relative differences in activities of transaminases (AST, ALT) obtained in this research can be explained as a sublethal effect of toxicants, as presented in some earlier papers (Asztalos et al., 1988, 1990). The changes obtained in the concentration of urea could be caused by a gill dysfunction (Lockhart and Metner, 1984), since the urea is excreted mainly through the gills (Smith, 1929). The differences in the concentration of creatinine in certain periods of the year could be related to differences in spawning activities at the two analysed localities, and the differences observed in November could be the consequence of a kidney dysfunction (Lockhart and Metner, 1984). The differences in the concentration of proteins are hard to explain due to their different functions in the fish blood (Sandnes et al., 1988).

The values of the HSI, the diet, the length-weight relationship and the condition factor are all being used as indicators of the population condition in various papers. While the differences in the values of the HSI in this paper can be attributed to differences in the reproductive cycle as well, the differences in diets and length-weight relationships are probably caused by the influence of toxicants. However, Koss et al. (1986) cite that the field sampled pike did not show any changes in the HSI values and length-weight relationship under the influence of xenobiotics. The research results obtained for *Thymalus arcticus* indicate that the diet disturbances and growth reduction occur under the influence of toxicants (McLaey et al., 1987), while Hoque et al. (1998) cite that the HSI represents a more sensitive indicator of pollution than the Fulton's condition factor.

All parameters analysed in this paper indicate that the sublethal pollution led to changes both in biochemical parameters and in the diet and length-weight

relationship. Unfortunately in this case, this was not confirmed by a chemical analysis of water, although it was visible at the upstream locality, while at the downstream locality, which was constantly loaded with wastewater from the city collector and the paper mill, it was probably caused by water level and water temperature. This research showed that the analysed parameters are sensitive to water pollution, and also that their changes due to the pollution were reversible, i.e. that they returned to the normal ranges after the cessation of the sublethal pollution. Similar observations of the reversibility in the enzyme activity (AST, ALT, ALP) in the sera and of gill lesions under the influence of sublethal concentrations of copper sulphate were observed in experiments conducted on the carp as well (Karan et al., 1998).

This research showed that the relative increase of the analysed parameters can point to a deterioration of environmental conditions. Also, such an approach is rather complex, having in mind the relation of significant changes in analysed parameters to changes in exogenous (photoperiod, water temperature) and endogenous (diet, reproductive cycle) factors during the annual cycle. The presented data also point to the necessity of a comparative monitoring of biochemical blood parameters and pathological changes in the tissue (gills, kidney, liver), in order to use these biochemical parameters as indicators of organ dysfunction.

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**Nitrite causes hematological disturbances in
*Colossoma macropomum***

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Abstract

The effects of acute exposure to nitrite (different concentrations during 24h) on physiological aspects of respiration of *Colossoma macropomum* (tambaqui) were estimated. The physiological stress was monitored following changes in tissue metabolic rates and hematological parameters. Increases in plasma glucose and lactate levels, and a decrease in intracellular pH indicate activation of anaerobic metabolism as the result of an impairment of oxygen unloading at tissue levels. However, the expected increase in methaemoglobin levels could not be correlated with a decrease in tissue metabolic rates, suggesting activation of cellular oxidative processes.

Introduction

Tambaqui is a native fish distributed across the Amazon and the Orinoco Hydrographic Basins. It is amongst the most important commercial fish species in Brazil. In aquacultural facilities high levels of nitrite can be found causing severe physiological disturbances. Nitrite causes, among other effects, the increase of methaemoglobin levels in fish, which is unable to bind oxygen, and so impairing tissue oxygenation. This work shows the effects of nitrite exposure on some physiological parameters of the species *Colossoma macropomum* (tambaqui).

Materials and Methods

Fish were obtained from Amazonfish Farm, near Itacoatiara City (AM). In the laboratory (Laboratory of Ecophysiology and Molecular Evolution – LEEM/COPE, INPA), the animals were acclimated over a 30 day-period before the beginning of the experiments. After this recovery period, fish were transferred to 4 experimental glass aquaria, with the following dimensions: 29 x 68 x 40 cm. Eight fishes were placed in each aquarium, which was filled with water and sodium nitrate at the following concentrations: 0 (control), 0.9, 2.7, and 3.6 mmol.l⁻¹ during 24h.

Blood was collected from the caudal vein into heparinized syringes and kept in ice. Hematological parameters (haematocrit, haemoglobin concentration, red blood cell counts, and corpuscular constants) were estimated by classical methods. Plasma sodium and potassium levels were estimated by flame photometry in a FC Celm 180 flame photometer. Plasma pH was measured using a glass micro-capillary electrode Radiometer G229A-Copenhagen, coupled to the pH meter PHM-73. Radiometer precision buffers were used to calibrate the electrode. Red cell pH was determined using freeze and thaw method. Methaemoglobin levels were estimated as described by Benesch *et al* (1973). Plasma glucose and lactate levels were estimated by enzymatic methods using commercial kits (Sigma Chem. Co. for lactate levels, and Doles® for glucose levels). Results are expressed as means ± SEM and the significance of the difference between treatments were estimated by ANOVA with a fiducially limiting of 5%.

Results and Discussion

Haematocrit values, haemoglobin concentration ([Hb]), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) of *C. macropomum* exposed to different nitrite concentrations under 24h are shown in Table 1. Haematocrit values (%) revealed a significant decrease in all tested nitrite concentrations. RBC counts were not different in any of the tested nitrite concentrations. On the other hand, MCV values decreased in the specimens exposed to nitrite at the concentration of 3.6 mmol.l⁻¹. Plasma Na⁺ levels, and extracellular pH, showed no change for all treatments. Plasma K⁺ levels were increased (P<0.05) in fish exposed to all treatments, which is uncommon and

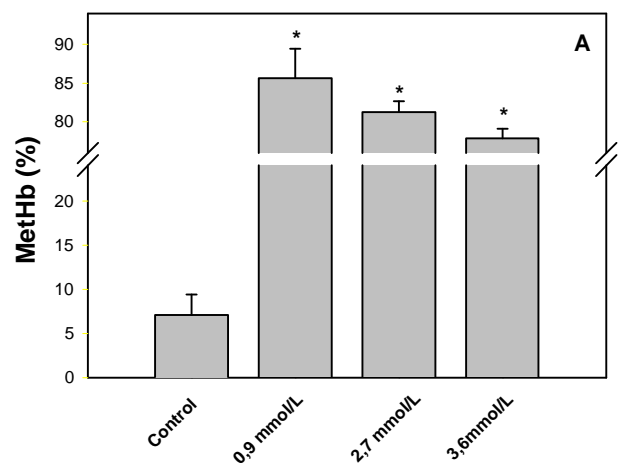
could only be explained as the result of cell damage. However, the analysis of *Astronotus ocellatus* (Paula-Silva *et al.*, 1996) under similar conditions showed no changes in plasma K⁺ levels. Intracellular pH decreased in fish exposed to sodium nitrite at the concentration 3.6 mmol.l⁻¹, suggesting proton accumulation inside the cell.

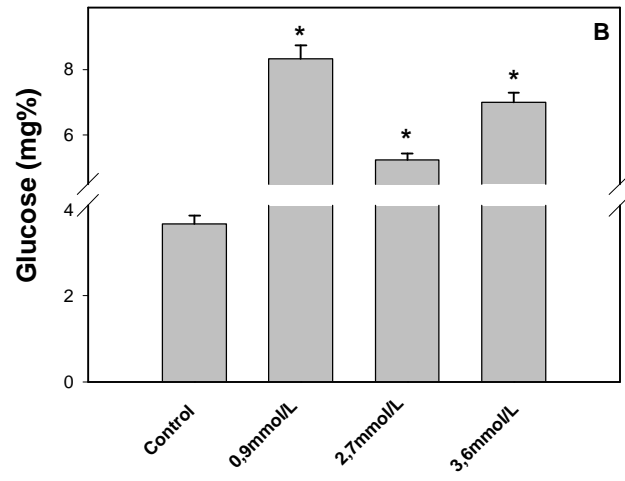
Table 1: Hematological parameters for *C. macropomum* exposed to different concentrations. Data are shown as mean ± SEM.

Nitrite levels (mmol.l ⁻¹)	Control	0.9	2.7	3.6
Ht %	22.62±0.76	21.56±0.49*	20.75±0.37*	20.00±0.27*
[Hb](g%)	5.37±0.18	5.97±0.22*	6.23±0.20*	5.19±0.09
RBCx10 ⁶ mm ⁻³	1.35±0.07	1.43±0.12	1.39±0.06	1.45±0.06
MCV(μm ³)	166.68±4.82	155.01±10.82	147.23±5.32	137.77±5.61*
MCH(pg)	24.07±0.42	27.16±1.53*	29.84±1.38*	26.12±0.53*
MCHC(%)	69.56±2.92	60.87±9.89*	50.43±3.45*	52.94±2.45*
Na ⁺ mEq.L ⁻¹	139.25±2.51	144.87±1.81	142.25±2.10	143.38±1.89
K ⁺ mEq.L ⁻¹	3.57±0.19	4.44±0.25*	4.20±0.27*	3.95±0.26*
pHe	7.55±0.032	7.61±0.058	7.62±0.047	7.74±0.036
pHi	6.88±0.03	6.81±0.04	6.82±0.04	6.76±0.038*

* Indicates significant difference (P<0.05) from control.

MetHb raised about 75% in all tested concentrations (fig.1A), indicating a depression in capacity of oxygen transport in fish blood. Plasma glucose levels (fig.1B) and lactate (fig.1C) increased in all tested concentrations.





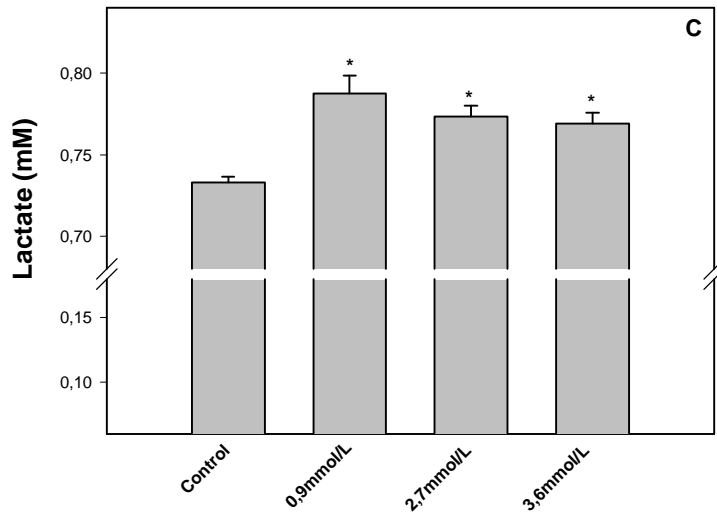


Figure 1: Plasma metHb (A), glucose (B) and lactate (C) changes of *C. macropomum* exposed to nitrite. * Indicates significant difference ($P<0.05$) from control.

Several authors reported the physiological effects of the nitrite on aquatic organisms (Williams & Eddy, 1986; Jensen, 1996; Knudsen & Jensen, 1997; Paula-Silva *et al.*, 1996; Duncan *et al.*, 1998; Costa *et al.*, 1999). Nitrite oxidizes haemoglobin to methaemoglobin (MetHb), which is unable to bind oxygen. Increased levels of MetHb cause an impairment of oxygen transfer that results in severe hypoxia and damage to several tissues. Ultimately, increased MetHb result in fish death (Arillo *et al.*, 1984). According to Hanson & Grizzle (1985), fish exposed to intermediate levels of nitrite become more vulnerable to bacterial diseases. They also suggest that nitrite may suppress fish immune system.

When exposed to nitrite, carp presents a KCl efflux from red blood cells, which is followed by an osmotic loss of water, reducing the volume of the red cells (Jensen, 1990, 1992). This fact was also observed in the present work, since animals exposed to sodium nitrite at the concentration of 3.6 mmol.l⁻¹ presented a decrease in their cellular volume (MCV). Nitrite effect in the fish tissues and their immune system responses are very similar to the effects of bioaccumulation of a pollutant, not only in the plasma, but also in the gills, liver, brain, spleen, muscle, etc. (Thurston *et al.*, 1978; Margiocco *et al.*, 1983; Scarano *et al.*, 1984). Hyperventilation and shivering signals were observed in the tambaqui exposed to nitrite. Such responses could not be linked to hypoxic conditions of the environment since the aquaria were aerated during the whole experimental stage; this may be the reason why lip extension, which is common in tambaqui exposed to hypoxic situations, was not observed in the present work.

Acknowledgements

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**PARALYTIC SHELLFISH TOXINS AND X
ENOBIOTIC METABOLISING ENZYMES
IN ARTIFICIALLY INTOXICATED
ATLANTIC SALMON (*SALMO SALAR*)**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Paralytic shellfish toxins (PSTs) are a group of potent neurotoxins produced by toxic strains of dinoflagellates. Exposure of fish to such toxins can result in fish kills (White, 1977) and other deleterious effects in marine food webs (Geraci et al. 1989). A greater understanding of the precise fate of PSTs in marine organisms is therefore required. Studies in this field to date have focussed on the dynamics of PST depuration from bivalve molluscs (Bricelj and Shumway 1998), yet little is known of the mechanisms responsible for eliminating these toxins from fish. It has been suggested that xenobiotic metabolising enzymes (XMEs) may be involved in the metabolism of algal toxins (Washburn et al. 1996). Intra-peritoneal (ip) exposure of salmon (*Salmo salar*) to saxitoxin (STX) has been shown to effect the induction of cytochrome P-4501A, a phase I XME, as measured by 7-ethoxyresorufin *O*-deethylase activity (Stagg et al. 1998).

Investigated here is the potential role of the phase II XME glutathione *S*-transferase (GST) in PST metabolism. GSTs catalyse the conjugation of reduced

glutathione (GSH) to electrophilic centres on substrates. This activity is inducible on exposure of the organism to the substrate.

The objective of this study was to determine the induction response of GST activity in Atlantic salmon exposed to PSTs by injection. This was achieved by three injections of post-smolts with PSTs (STX [2 :g/kg] or an extract from a cultured toxic dinoflagellate [*Alexandrium fundyense* CCMP 1719, 2.26 :g/kg] in physiological saline over 21 days. For control purposes fish were exposed to physiological saline or an extract from a non-toxic dinoflagellate (*Scrippsiella trochoidea* NEPCC 15).

Hepatic GST activities were found to differ significantly between treatment groups ($P < 0.001$). Fish exposed to both saxitoxin and a toxic dinoflagellate extract containing a number of PST analogues demonstrate nearly two-fold induction of activity over controls (Fig. 1).

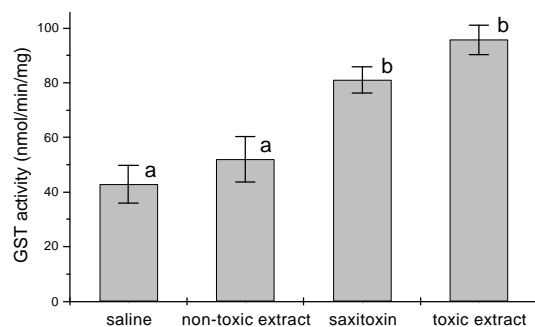


Fig. 1. Hepatic glutathione *S*-transferase activity of Atlantic salmon exposed over 21 days to multiple ip injections of: physiological saline, saxitoxin and extracts of toxic (*Alexandrium fundyense*, CCMP 1719) and non-toxic (*Scrippsiella trochoidea*, NEPCC 15) cultured dinoflagellates. Data are means \pm SE, $n = 10$. ab Groups with different notation are significantly different ($P < 0.05$).

The major GST isoform in salmon livers has been shown to be homologous to mammalian B class GST (Dominey et al. 1991). Antibodies raised against rat B

class GST (obtained from John Hayes, Dundee University) detect a single protein band in protein fractions from both rat (positive control) and salmon livers (Fig. 2a). Rat B class GST was estimated at 26.8 kDa in size, while the 'B class GST-like' protein detected in salmon samples was 27.6 kDa.

Variation in salmon 'B class GST-like' protein was inferred from immuno-peroxidase stained dot blots by quantifying band intensities on scanned blots. This analysis showed that fish injected with a toxic dinoflagellate extract not only demonstrate the highest levels of hepatic GST activity (Fig. 1), but also contain significantly elevated levels of 'B class GST-like' protein (Fig. 2b). This suggests that elevated levels of the major GST protein in salmon livers may be partly responsible for inducing hepatic GST activity in salmon exposed to PSTs.

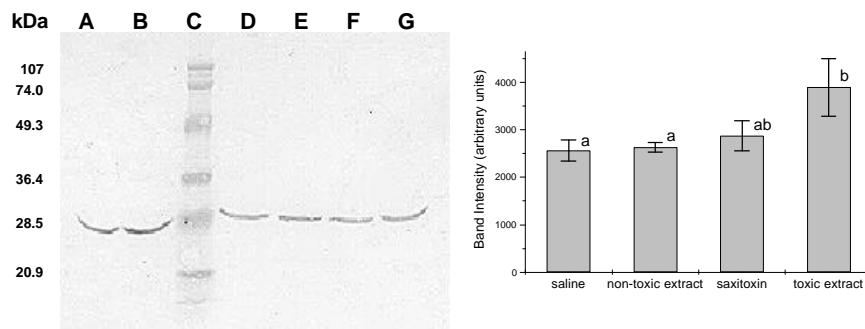


Fig. 2a. Western blot probed with anti-rat B class glutathione *S*-transferase antibodies and visualised by immuno-peroxidase staining. A-B) protein fractions from rat liver run as a positive control at 40 and 80 :g protein loadings respectively. C) pre-stained BioRad low range size marker. D-G) protein fractions from salmon livers (40 :g protein). **2b.** Band intensities from dot blots probed and visualised in the same manner. Samples (200 :g protein) are from salmon treated as per Fig. 1. Data are means \pm SE, n = 10. ab Groups with different notation are significantly different (P<0.05).

In the fish model chosen, artificial intoxication with PSTs (saxitoxin or extracts from toxic dinoflagellates) results in induction of GST activity in the liver. This

may be caused by increased protein expression. Such induction suggests that this enzyme system may play a role in the metabolism of this group of algal toxins. Exposure of fish to PSTs from toxic dinoflagellate blooms may therefore result in a greater capacity of the liver to conjugate GSH to absorbed toxins.

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**LIVER HISTOPATHOLOGY AND PAH BILE METABOLITES FOUND
IN ENGLISH SOLE (*PLEURONECTES VETULUS*) COLLECTED FROM
AN AREA ASSOCIATED WITH ALUMINUM SMELTING**

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Abstract

Bioindicators of sublethal effects associated with aluminum smelting activities were examined in the bottom-feeding flatfish, English sole (*Pleuronectes vetulus*). Histopathological analysis of liver samples from fish collected near the aluminum smelter revealed neoplastic lesions. The liver tissues from this site also exhibited a pathological iron storage disorder, hepatocellular hemosiderosis. Synchronous scan fluorescence spectrometry was used to measure pyrenol conjugate concentrations in bile. The PAH bile metabolite concentrations reflected sedimentary PAH levels and were associated with the adverse histopathological conditions. The presence of pyrenol conjugates indicates biological activation of PAHs that are known to cause cancerous lesions. Another contributing factor towards neoplasia may arise from oxidative stress and DNA damage resulting from reactive oxygen species. These oxyradicals may have been generated from the Fenton reaction catalysed by excess iron. Additional research is underway to determine the nature of the above linkages and their roles in carcinogenesis.

Introduction

Marine environments near industrial and urban centers are contaminated with a wide range of chemicals that may be transformed, either biologically or chemically, into new potentially toxic compounds (Malins and Haimonot, 1981). Research has demonstrated a positive link (by association) between the presence of certain xenobiotic chemicals in sediments, seawater or food organisms and the onset of sublethal effects in demersal (resident) fish species. Sublethal toxicity tests may make it possible to detect incipient effects on fish and to estimate threshold concentrations. However, no single approach to the problem of biological effects monitoring can be fully satisfactory. Some methods seem more useful than others, but greatest insights have usually been obtained by multidisciplinary efforts, often those in which pathology has been allied with biochemistry, then augmented by chemical analyses of tissue and environmental samples (Adams et al., 1996).

This report examines a suite of selected stress responses on English sole (*Pleuronectes vetulus*) residing in area effected by aluminum smelting activity. A toxicological approach for early sublethal stress effects on this flatfish species was examined using the following levels of biological organization: histopathological, by diagnosis of fish liver for idiopathic lesions; histochemical, using Perls' method (Prussian blue) for ferric iron to demonstrate hemosiderosis; and bile metabolites, measured as 1-pyrenyl glucuronide (1-pyrenyl- β -D-glucopyranosiduronic acid) equivalents using synchronous scan fluorescence spectrometry (SFS).

Materials and Methods

Field collection

English sole, *Pleuronectes vetulus*, were collected on board the C.C.G.S. Vector using a beam trawl from Kitimat Harbor, the Kitamaat Village and the Kildala, areas, located on the north coast of British Columbia, Canada, in 1994 to 1997. Only female English sole greater than 26 cm and males greater than 24 cm were sampled to insure that all fish were 4 years of age or older (Garrison and Miller 1982, Harry 1959). Five to thirty English sole from each of the sampled populations were sacrificed, measured for condition factors, liver tissue excised and fixed in Dietrich's fixative for histopathology. Bile was collected and stored at -80°C for measurement of polycyclic aromatic hydrocarbons (PAH)

conjugated metabolites. The otoliths were removed and stored in glycerin/ethanol for subsequent determination of age (Chilton and Beamish 1982).

Liver histopathology

Fixed liver tissues were washed in water, dehydrated in an ethanol series, cleared in toluene and embedded in paraffin (Paraplast). Skip serial sections were cut at 5 µm, mounted on slides and stained with Gill's haematoxylin and eosin (Humason 1979) for general histology. Liver tissue was also stained with Perls' method (Prussian blue) for ferric iron (Pearce 1972). The slides produced were scored blind (without information of the particulars of the collection site), and liver abnormalities were classified using nomenclature consistent with the system of Myers et al., (1987).

PAH conjugates in bile

PAH metabolite conjugates in bile were measured by synchronous-scan fluorescence spectrometry (SFS) at Simon Fraser University following the method of Ariese et al., (1993). The samples of bile were diluted 1/500 with a 1:1 HPLC grade ethanol and HPLC water solution (Fisher Scientific Ltd.). The fluorescence response was measured on a Perkin Elmer Luminescence spectrophotometer LS-50 with FL data manager operating on an IBM-PC compatible computer. Both excitation and emission monochromators were scanned synchronously with a fixed wavelength difference of 37 nm. The area of the fluorescence emission response was measured from 335-356 nm on the emission monochromator. A six point linear external calibration curve of 1-hydroxypyrene was used to calibrate the fluorescence spectrophotometer. The fluorescent emission of the 1-hydroxypyrene standards was measured from 340-361 nm. A conversion factor of 2.2 was applied to account for the difference in fluorescence yield between 1-hydroxypyrene and its conjugate 1-pyrenyl glucuronide, which is the major metabolite conjugate of pyrene in the bile. Protein analyses of fish bile were done by the method of Lowry et al., (1951) using a Bausch and Lomb Spectronic 20 spectrometer.

Results

Catch size (or sample size) for English sole (*Pleuronectes vetulus*) were all well below optimum at each site due to limited availability and ship time. For both 1994 and 1997, catch sex ratios were not equal or consistent. Fish age differed between the collection sites and year, with a general trend of younger fish caught in 1994 compared to 1997 and an overall age range of 3 to 10 years (Table 1).

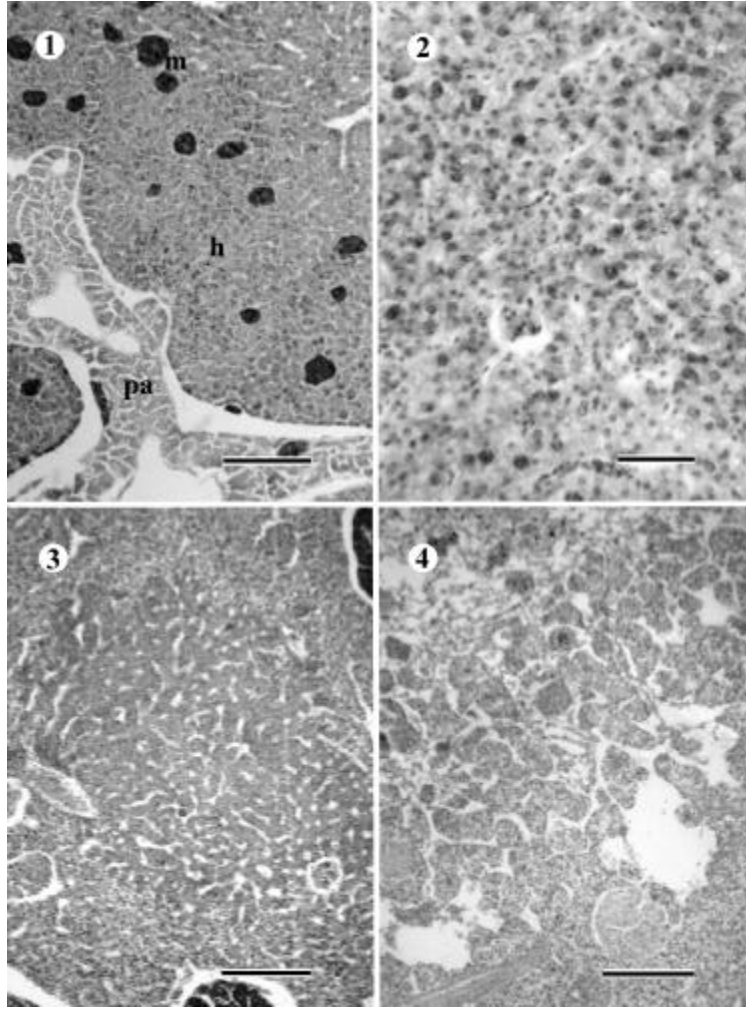
Table 1: Catch data and liver lesion prevalence for English sole (*Pleuronectes vetulus*) collected in 1994 and 1997 from areas influenced by aluminum smelting activity, Kitimat Harbor and Kitamaat Village, and a reference site, Kildala Arm.

Sample Site	Sample Size and Sex	Age (years) mean \pm s.d.	idiopathic liver lesion (%)	hepatocellular hemosiderosis (%)
Kildala 1994	5 males 10 females	4 \pm 1	7.2	33
Kildala 1997	1 male 4 females	7 \pm 3	0	40
Kitimat 1994 Harbor	15 males 4 females	4 \pm 1	21	87
Kitimat 1997 Harbor	1 male 9 females	6 \pm 2	30	70
Kitamaat 1994 Village	11 males 19 females	5 \pm 2	30	79
Kitamaat 1997 Village	4 males 6 females	7 \pm 3	30	70

Liver histopathology

English sole with normal liver tissue were found at all sampling sites. Parasites were present in liver samples collected from all the study sites examined. The most common was the plasmodial form of the myxosporean genus *Myxidium* (Butschli), family *Myxidiidae* (Thelohan), found within the bile ducts and ductules. The helminths, acanthocephala or nematoda, were occasionally present either externally or internally. Associated with these infections were inflammatory responses, resulting in the formation of granulomas.

Five types of idiopathic lesions were observed in fish collected from the vicinity of the aluminum smelter. These lesions were: nonspecific necrotic lesions; two types of intracytoplasmic storage disorders, hepatocellular hemosiderosis (Fig. 1) and variable hydropic vacuolation; nuclear pleomorphism and megalocytic hepatitis (Fig. 2); foci of cellular alteration, clear cell, eosinophilic and basophilic foci (Fig. 3); and the neoplasms, liver cell adenomas and hepatocellular carcinomas (Fig. 4).



- Fig. 1: Hepatocellular hemosiderosis shown as dark pigments (h) within the hepatocytes. m = melanomacrophages, pa = pancreatic acini. Perls' iron. Bar = 100 μ m.
- Fig. 2: Megalocytic hepatitis. Note the enlarged nuclei scattered throughout the parenchyma. H&E. Bar = 50 μ m.
- Fig. 3: Basophilic foci. Note the discrete nodular center with minimal compression of the surrounding parenchyma. H&E. Bar = 100 μ m.
- Fig. 4: Hepatocellular carcinoma. Note the atypical cellular morphology with significant compression of the adjacent tissue. H&E. Bar = 100 μ m.
-

In 1994, idiopathic liver lesions were observed in English sole collected from all three sites. Prevalences of lesions, however, were found to be higher in fish collected from the Kitamaat Village (30%) compared to Kitimat Harbor (21%) and Kildala Arm (7.2%) (Table 1). In 1997, both the Kitimat Harbor and Kitamaat Village population revealed a 30% liver lesion incidence, whereas all reference population liver samples were normal. When samples were tested for the iron storage disorder, hepatocellular hemosiderosis, percent differences between the reference and study populations were observed. In 1994, 79 and 87% of the Kitamaat Village and Kitimat Harbor population exhibited this condition compared to 33% of the Kildala population. The 1997 results were similar, with 70% of the fish from Kitimat Harbor and Kitamaat Village affected compared to 40% from Kildala Arm.

PAH conjugates in bile

Lowest levels of pyrenol conjugate concentrations in English sole bile were found in fish collected from the Kildala site, ranging from 13.9 ± 2.8 to 15.8 ± 5.4 μ g 1-pyrenyl glucuronide/mg bile protein for samples collected in 1994 and 1997 respectively. These values were found to be on or slightly below detection limits. Higher levels were observed in the fish bile samples obtained from the Kitimat Harbor and Kitamaat Village ranging from 38.1 ± 18.7 to 210.8 ± 164.4 μ g 1-pyrenyl glucuronide/mg bile protein, depending on the site and year of collection. In general, bile pyrenol conjugate concentrations showed a constant and low trend for the fish collected from the reference site, whereas fish collected near and adjacent to the aluminum smelter, particularly Kitamaat Village populations, showed high, but declining levels (Table 2).

Table 2: PAH metabolite conjugates in English sole (*Pleuronectes vetulus*) bile expressed as μg 1-pyrenyl glucuronide equivalents / mg protein, measured by synchronous-scan fluorescence spectrometry (SFS) (Ariese et al., 1993), for fish collected near an aluminum smelter (Kitimat Harbor and Kitamaat Village) and a reference site (Kildala) in 1994 and 1997.

Sample site	μg 1-pyrenyl glucuronide equivalents / mg protein*	
	1994	1997
Kildala	13.9 \pm 11.1 (15)	15.8 \pm 5.4 (5)
Kitimat Harbor	54.9 \pm 19.9 (19)	39.5 \pm 16.9 (10)
Kitamaat Village	210.8 \pm 164.4 (30)	38.1 \pm 18.7 (10)

*values expressed as averages \pm standard deviation (n=sample size)

Discussion

English sole collected from Kitimat Harbor and Kitamaat Village had higher incidences of preneoplastic and neoplastic liver lesions compared to sole collected from the Kildala. Studies of flatfish populations from non-urban reference sites in Puget Sound, Washington have shown no evidence of idiopathic liver lesions (Tetra Tech, 1987). In contrast, studies of wild fish populations from polluted environments have shown a higher prevalence of hepatic lesions (Harshbarger and Clark 1990). For a number of areas in Puget Sound (eg. Eagle Harbor and Duwamish Waterway) (Malins et al., 1984 and 1988) and Vancouver Harbor (Goyette et al., 1988) a positive correlation has been shown between sediment-associated polycyclic aromatic hydrocarbons (PAHs) and prevalences of several categories of idiopathic liver lesions in English sole. Similar to these studies, prevalences of English sole with idiopathic liver lesions appear to be dependent on location of capture with high prevalences (~30%) of preneoplastic and neoplastic lesions found in English sole collected from both Kitimat Harbor and Kitamaat Village. A possible positive correlation may also exist with the observed PAH metabolite levels found in their bile samples and the observed incidences of liver lesions. Only preneoplastic (megalocytic hepatitis and all foci of cellular alterations) and neoplastic (liver cell adenomas and carcinomas) lesions were considered in calculating these frequencies. Other idiopathic lesions were omitted due to the uncertainty in their relationship to exposure to xenobiotic, carcinogenic chemicals.

The intercytoplasmic storage disorder, hepatocellular hemosiderosis, was prevalent in a high percentage of sole collected from Kitimat Harbor and Kitimaat Village compared to those collected from the reference site, Kildala. Hemosiderosis suggests an underlying metabolic disorder characterized by excessive accumulation of intracytoplasmic iron within the hepatocytes (Myers et al., 1987). When this condition is experimentally induced in rats the frequency of developing hepatocellular carcinomas with exposure to xenobiotics increases. DNA damage and mutagenesis brought about by iron are likely to occur by a Fenton-type mechanism that involves the generation of (i) hydrogen peroxide by the autooxidation of iron and (ii) hydroxyl radicals by the interaction of hydrogen peroxide with iron (Loeb et al., 1988). Recently Payne et al., (1998) has reported levels of DNA oxidative damage in trout collected from lakes receiving effluents from iron-ore mines and these authors suspect that the high environmental iron loading may catalyze reactions generating free radicals and DNA damage.

Recent studies in the Kitimat marine environment have attempted to establish a link between environmental concentration of contaminants and biological effects. The surprising result so far has been that the predicted acute toxicities of the sediments, based on their PAH concentrations, have not been observed in sensitive short term bioassays and benthic infaunal surveys. It has been proposed that the PAHs, which are produced predominantly by the aluminum smelter, are not readily bioavailable (Paine et al., 1996, Naes et al., 1998). The PAHs appear to be associated with soot particles released from the electrodes in the smelter (Naes et al., 1998, Cretney, unpublished). The partitioning of PAHs from soot particles to the water may be up to a 1000-fold less than that from natural organic carbon (Gustaffson et al., 1997). Our research has revealed PAH metabolites in bile from English sole. Thus, PAHs are bioavailable in Kitimat Arm, though at concentrations much less than would be expected from their concentration in the sediments (Paine et al., 1996). More study is required to determine whether the main source of the PAHs metabolites in the bile is soot carbon from the smelter or other sources, such as petroleum from marine traffic.

Conclusion

Due to the environmental importance of epizootics of neoplasia in benthic animals and their apparent association with mammalian carcinogens, considerable interest has arisen in studying genotoxic and carcinogenic responses in these organisms. There are numerous examples of hotspots, where

tumour incidences in fish have been correlated with raised concentrations of anthropogenic chemicals, but causal mechanisms are seldom established. This study, plus other available information from recent research in aquatic and medical toxicology, suggests that responses associated with xenobiotic metabolism, oxidative stress, and DNA damage may be mechanistically linked. Additional research is currently underway to determine the nature of these linkages, their roles in carcinogenesis, and the causative factors for the associated responses in aquatic organisms exposed to sediments of complex natures. Results from this study may also provide sufficient information towards the potential use of marine organisms as sentinels, that can provide early warning signals of potential threats to man.

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**DISPOSITION OF PCB DURING THE WINTER EMACIATION
OF THE ANADROMOUS ARCTIC CHAR**

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Introduction

The anadromous Arctic char *Salvelinus alpinus* (L.) undertake annual migrations to the sea in early summer, and usually reside in the sea for 40-50 days before returning to fresh water (Finstad and Heggberget, 1993). During the short seawater residence, individuals may double in body weight (Mathisen and Berg, 1968) and increase body lipid stores several fold (Dutil, 1986; Jørgensen et al., 1997a). In winter the food intake is minimal and stored lipids are mobilised to meet metabolic demands. When new excursions to the sea are to be undertaken, the lipid stores may be depleted (Boivin and Power, 1990; Jørgensen et al., 1997a; Jobling et al., 1998). Such seasonal variations in body lipid stores are characteristic for many high-latitude inhabitants.

Aquatic pollution is an increasing problem, and even in remote Arctic areas high levels of several pollutants have been detected (e.g. Hargrave et al., 1992; Muir

et al., 1992). Persistent organic pollutants (POPs) enter the fish primarily via the food (Sijm et al., 1992). In high-latitude environments, fish are consequently contaminated during the short season in which they eat. Deposition of POPs within the animal appears to be positively correlated with the localisation of non-polar lipids like triacylglycerols (TAG) (Kawai et al., 1988). Hence, in well fed, "fat" fish, a high proportion of the total POP burden is found in tissues containing the majority of the body lipids; i.e. in the muscle, skin and skeleton in the Arctic char (Jørgensen et al., 1997a). During periods of lipid mobilisation, there may be a redistribution of deposited POPs from adipose tissues toward vital organs like the liver, kidney and brain (Boon and Duinker, 1985; Jørgensen et al., 1997a). Periods of POP redistribution would therefore carry a risk of increased pollutant-associated stress.

In order to study these processes, a semi-field model has been developed. In this report we present the results from a pilot study which was conducted in order to reveal the validity of such an experimental approach.

Materials and Methods

The experiment was performed with a 21 immature, anadromous Arctic char (*Salvelinus alpinus*) from Halsvassdraget, located in Finnmark, northern Norway (70°N 23°E). A permanent fish trap located near the river mouth, catches all migrating fish. On the return to freshwater in late July fish were collected in the trap and transferred to a 3 m³, circular indoor tank supplied with unheated river water.

On August 15, 1996, length and weight of the fish were measured. Two weeks later all fish received an oral dosage of PCB (1.5 µg/gram fish weight) by force feeding of a gelatine capsule containing PCB solved in fish oil. The PCB administered constituted equal amounts of 4 congeners (IUPAC no. 101, 105, 153 and 180), purchased from Promochem AB (Ulricehamn, Sweden).

Until September 15, the fish were held in continuous light, after which the photoperiod was kept at 10 h light, 14 h dark until March 21, and thereafter increased to continuous light. This photoregime was used at the research station in order to give a "normal" parr-smolt development. In order to mimic the non-feeding overwintering in wild, anadromous char, the fish were not fed during this period.

On September 17, February 19 and May 28 seven fish were sacrificed at each date. The fish were weighed and dissected into the following parts: liver, kidney, brain and the remaining carcass (including muscle, gut, skin, head, skeleton and fins). All organs, except the brain, were divided into two parts, one for lipid analysis and one for PCB analysis. Due to the small size, the brain was only analysed for PCB.

Total lipids were analysed from homogenised samples by a mixture of methanol and chloroform, essentially as described by Bligh and Dyer (1959). The proportional amount of triacylglycerols (TAG) was quantified by HPTLC (high-performance thin-layer chromatography), using SiO₂ 60 pre-coated plates (Merck, Germany). The developing solvent system consisted of heptane : diethyl ether : acetic acid (80:20:1). After charring with copper acetate the separated lipid classes were quantified by a Camag TLC scanner 3 (Muttentz, Switzerland) at the wavelength 350 nm.

For the PCB analyses, samples were homogenized with acetone and thereafter hexane : acetone (3:1). After clean-up PCB was determined by gas chromatography, using a HPGC 5890 Series II equipped with a splitless injector, electron capture detector (ECD) and a HP-5 capillary column, (50 m x 0.20 µm x 0.11 µm). Nitrogen was used as carrier gas (42 ml/min). The injector and detector temperatures were 280°C and 320°C. The temperature program was: 60(1)-15-160(0)-1.5-270(20). Calculations were done on the basis of external (PCB 53) and internal standards with which the chromatograph had been calibrated.

One-way ANOVA (Statistica 5.1, StatSoft Inc., USA) was used to test differences between tissues/organs and sampling dates with regard to PCB concentrations and congener composition. A probability level < 0.05 was considered significant.

Results

Table 1 and 2 shows the weight of the carcass, liver and kidney, and the percentage of total lipids and TAG in these tissues and organs at the different sampling dates.

Table 1 Tissue and organ weights (g) \pm s.e.m. (standard error of a mean) in immature anadromous Arctic char sacrificed at different dates throughout the winter.

Month	n	<i>Carcass</i>		<i>Liver</i>		<i>Kidney</i>	
		Weight	<i>s.e.m.</i>	Weight	<i>s.e.m.</i>	Weight	<i>s.e.m.</i>
September	7	355	19	3.9	0.20	3.3	0.29
February	7	309	11	3.3	0.07	2.9	0.13
May	7	260	15	3.1	0.16	2.9	0.22

All organs and tissues decreased in weight throughout the winter. On the average, the carcass lost 27 % of its weight from September to May, whereas the weight loss of the liver and kidney were slightly less (20 and 12 %, respectively).

Table 2 The percentage of total lipid and TAG (triacylglycerol) in different tissues and organs in anadromous char sacrificed at different dates throughout the winter. Values shown are means (n=7) \pm s.e.m. (standard error of a mean).

Month		<i>Carcass</i>		<i>Liver</i>		<i>Kidney</i>	
		%	<i>s.e.m.</i>	%	<i>s.e.m.</i>	%	<i>s.e.m.</i>
September	Tot.lip	3.4	0.6	13.9	2.4	3.6	0.3
	TAG	2.4	0.6	8.1	1.6	1.9	0.3
February	Tot.lip	1.8	0.13	4.1	0.4	2.4	0.07
	TAG	1.1	0.11	1.1	0.3	0.6	0.12
May	Tot.lip	1.2	0.08	2.9	0.16	2.0	0.10
	TAG	0.5	0.07	0.1	0.03	0.1	0.03

The lipid content decreased in all tissues and organs during the winter. The greatest reduction was seen in the liver, where the amount of lipid on average decreased 83% from September to May. In the carcass, 74% of all the lipids were utilised between September and May. Quantitatively, most lipids were lost from carcass (muscle, skin, bone, fins, and head) during the winter due to the high proportional weight of these tissues (98% of total fish weight). In September more than 50% of the total lipids in all tissues and organs was TAG.

In May, the proportion of TAG had decreased to 4.0, 3.5 and 5% of the total lipids in the carcass, liver and kidney, respectively.

In May, the total body burden of PCB was approximately 80 % of that found in September. No significant differences were found in the proportional amount of the four congeners, neither between organs, nor between sampling dates ($p < 0.05$). Figure 1 shows the concentration of PCB in carcass, liver, kidney and brain in September, February and May.

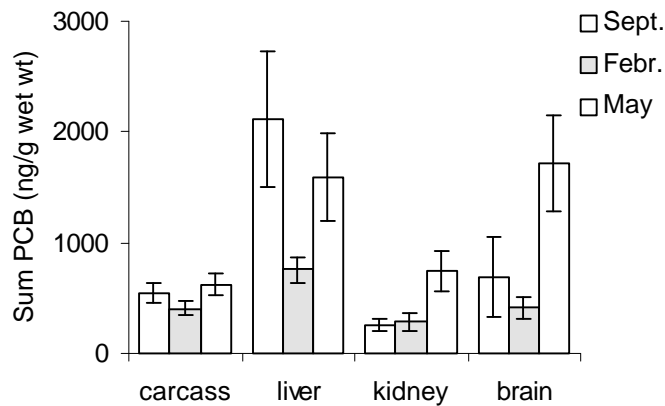


Figure 1 Concentration of PCB (sum of 4 congeners) in different tissues and organs the char sacrificed in September, February and May. Values shown are sample means ($n=7$) \pm s.e.m. (standard error of a mean).

In the carcass, the PCB concentration remained relatively stable during the winter. In the liver a high concentration of PCB was recorded in September, after which there was a significant decrease until February ($p < 0.05$) and a subsequent, significant ($p < 0.05$) increase until May. In the kidney, the PCB concentration did not change from September to February, after which there was an increase (but not significant) until May. In the brain PCB concentration did not differ significantly between September and February, but there was a significant increase (approx. 4 fold) between February and May ($p < 0.05$).

In figure 2 the individual concentration of PCB in the brain is plotted against the percentage of TAG in the carcass. As evident in the figure, there appeared to be a marked increase in the brain PCB concentration when the percentage of TAG decreased below 1% of the carcass wet weight. A similar increase, although not so dramatic, was seen in liver and kidney.

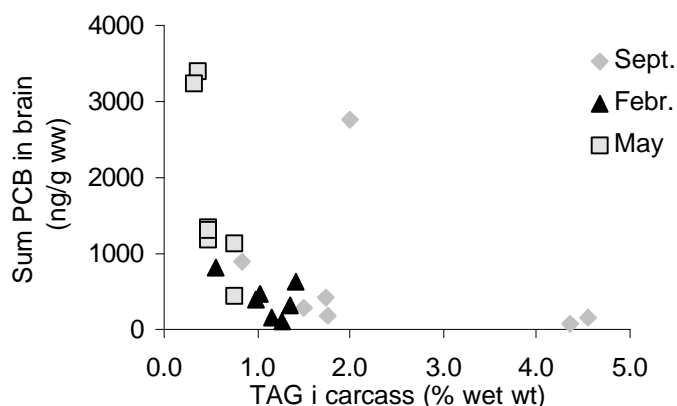


Figure 2 PCB concentrations in brain plotted against the concentrations of triacylglycerol (TAG) in carcass for individual fish, sampled in September (◆), February (▲) and May (■).

Discussion

The changes in body weight, organ weights and lipid status in the char held in captivity throughout the winter were comparable to the changes occurring in their wild counterparts overwintering in the lake (Jørgensen et al., 1997a; Jobling et al., 1998). This indicates that the energy expenditure of wild char overwintering in indoor tanks is comparable to that in the wild. These results confirm the applicability of this model for realistic studies on POP toxicokinetics in relation to the anadromous life strategy of the Arctic char.

The 20 % reduction in the total body burden of PCB between September and May indicated a very long half-life (>600 days) of the PCB mixture used in the present experiment. This is in accordance with the very slow elimination rate of PCB congeners with high chlorine content previously found in fish (e.g. Niimi

and Oliver, 1983). All four PCB congeners used in the present experiment were either pure phenobarbital (PB) type inducers (PCB 101, 153 and 180) or mixed (both PB and 3-methylcholanthrene) type inducer (PCB 105) (McFarland and Clarke, 1989). The metabolisation and excretion rates of these congeners were therefore expected to be relatively similar. This was also the case, in that the temporary changes in the body burden from September to May did not differ between the congeners.

In September the concentrations of PCB were fairly similar in carcass, kidney and brain, as were also the concentrations of TAG in carcass and kidney. At the same time, the liver concentrations of both the PCB and TAG were 3 to 4 times higher than in the other organs. In February PCB and TAG concentrations were comparable for all tissues and organs. In accordance with previous findings in fish (Monod and Keck, 1982; Kawai et al., 1988; Kamman et al., 1990; Jørgensen et al., 1997b), the tissue distribution of PCB in September and February in the present study seemed to be related to the tissue concentration of non-polar lipids (i.e. TAG), at least in the carcass, liver and kidney.

From February to May the PCB concentration increased only slightly in the carcass, whereas a 2 to 4 times increase were seen in the liver/kidney and brain, respectively. In May, the concentration of TAG were substantially lower in liver and kidney (0.1 %) than in carcass (0.5 %), and the change in the tissue distribution of PCB between February and May could therefore not be attributed to corresponding changes in the affinity for PCB between tissues and organs.

Temporary changes in the tissue concentration of PCB in starving fish depend on the relationship between the rates of PCB loss and the reduction of the organ weight. In the present study there was on average 30 % decrease in both the weight and PCB content in carcass from September to May. The fairly stable PCB concentration indicates that there were no apparent changes in the mobilisation of PCB from the carcass during the course of the experiment.

The increased concentration of PCB in liver, kidney and brain from February to May was not accompanied by a corresponding decrease in the weights of these organs. This indicates that there must have been a net input of PCB to these organs from February to May. Hence, we would expect an increased mobilisation of PCB from the carcass in this period, an assumption that is not supported by the data. More research is needed to reveal the mechanisms underlying the disposition of POPs after long-term fasting in fish.

The mean concentration of PCB in the brain increased approximately four-fold from February to May. When the brain PCB concentration of individual fish were plotted against carcass TAG concentration, there seemed to be an exponential increase in the brain PCB concentration when the TAG concentration in the carcass decreased below 1 %. Apparently, this pattern caused large inter-individual differences in the concentration of PCB in vital organs along with small differences in the degree of emaciation among these individuals. The dramatic increase in the brain PCB concentration, and to a certain extent also in the liver and kidney, at the end of the winter in some individuals must have implications for the toxicological potential of deposited POPs. Since at least some of the toxic effects of halogenated aromatic hydrocarbons such as PCB are mediated through the cytosolic aryl hydrocarbon (Ah) –receptor (Safe, 1990), dose-response relationships depends on the concentration of the toxicant in cells containing the Ah-receptor. In general, Ah-receptors have not been found in skeletal muscle cells (Guengerich, 1993), whereas they are prevalent in both liver, kidney and brain cells in fish (Husøy et al., 1994; Andersson and Goksøyr, 1994). Temporary differences in the tissue distribution of POPs are therefore thought to affect the toxic potential of a certain body burden of POPs. Accordingly, a higher hepatic CYP1A activity was previously found in starved char than in fed charr due to a higher liver concentration of PCB in the former fish (Jørgensen et al., 1999).

In conclusion, the results in the present experiment indicate that the anadromous Arctic char are more vulnerable to negative effects associated to POPs in the late winter/spring and that there are marked differences among individuals in a population in the risk associated with toxicant burdens. The long half-life of POPs with high lipophilicity and temporary emaciation of many Arctic species probably makes them extra sensitive to POPs. In the anadromous Arctic char, winter emaciation coincides with the preparatory changes taking place prior to a new seaward migration and the present result aim for more research on possible effects of POPs on smoltification and seawater performance in this species. The present experimental model appears to be unique in that carefully controlled and ecologically realistic, contamination experiments can be performed with fish displaying natural traits regarding their physiology and body composition. New, comprehensive studies have consequently been undertaken to access 1) PCB disposition in relation to temporary changes in the localisation, content and composition of lipids, 2) dose- and time-dependent biological responses, and 3)

effects on the smoltification process and seawater performance in the anadromous Arctic char.

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**EFFECTS OF SEDIMENT-ASSOCIATED TRI-N-ORGANOTIN
COMPOUNDS ON THE OSMOREGULATION
OF FRESHWATER-ADAPTED 0-GROUP
EUROPEAN FLOUNDER, *PLATICHTHYS FLESUS* (L.)**

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Abstract

Chronic (5 weeks) exposure of freshwater-adapted European flounder, *Platichthys flesus* (L.), to environmental concentrations of sediment-associated tri-*n*-butyltin chloride (TBTCI) and tri-*n*-phenyltin chloride (TPhTCI) caused significant changes to hydromineral fluxes and membrane permeability, mechanisms that maintain osmotic homeostasis. The half-time of exchange of tritiated water (THO) in TBTCI- and TPhTCI-exposed fish was significantly increased during the first 2 weeks of the experiment and then decreased steadily, eventually reaching the level that the control group had constantly maintained throughout the experiment.

This change in apparent water permeability was accompanied by a significant decrease in diffusional water flux across the membranes. Passive Na⁺-efflux across the gills was increased significantly but effluxes in the control group were near constant over the same time span. Drinking rates in the organotin groups increased significantly while the rate of urine production did not change. This led to an increased net water balance in the organotin groups and consequently to a significant reduction of the blood osmolality of both organotin groups when compared to a control. There would appear to be a metabolic cost attached to the changes produced by exposure to organotin compounds which are manifested as a minimal increase in body length compared to the controls.

Introduction

Following the partial ban on the use of organotin-based anti-fouling paints on boats and maritime equipment in most industrialised countries, water concentrations of organotin compounds have dropped dramatically, albeit with hotspots remaining in areas of intense shipping activity (Waite *et al.*, 1996). However, there is an increasing amount of evidence to show that organotin compounds are persistent in marine and freshwater sediments, which can act as reservoirs and sources for the secondary introduction of organotins to the environment (Waldock *et al.*, 1990; Langston & Burt, 1991; Watanabe *et al.*, 1995; Harris *et al.*, 1996).

Despite this wealth of data there are few studies on the effects on benthic organisms exposed to environmental concentrations of organotin compounds in sediments; the most recent of these being the studies by Krone *et al.* (1996), Rouleau *et al.* (1998) and Werner *et al.* (1998). Although *in vivo* studies by Chliamovitch & Kuhn (1977) and Pinkney *et al.* (1989) have shown that high concentrations of organotins in aqueous suspension disrupted osmoregulation in euryhaline fish, the present study is the first to investigate the effect of environmental concentrations of sediment-bound organotin compounds on a benthic euryhaline fish.

The aim of this study was to detect and quantify any significant effects of chronic exposure of 0-group flounders to sediment-associated Tri-*n*-butyltin chloride (TBTCI) and tri-*n*-phenyltin chloride (TPhTCI) on osmoregulation that might affect the viability of such juvenile fish in terms of their ability to fully exploit a euryhaline environment. In addition to the physiological measures of the effects of organotin exposure, at the level of the whole organism, the metabolic cost was investigated by following the length increase of experimental and control groups.

Material and Methods

0-group flounders (0.4 - 1.8g) were caught at Woodmill, River Itchen, Southampton (Hutchinson & Hawkins, 1993) and kept in a 3,500 litre glass-fibre fish-farming tank in the hatchery at the Southampton Oceanography Centre, that was shielded from direct sunlight and rain by a roof, but exposed to natural temperature fluctuations and light/dark cycles. Prior to experiments, the

fish were sampled from the stock populations and acclimated to tap water at a temperature of 15°C and a light/dark regime of 12 hours on and 12 hours off for at least 2 weeks.

Fish were fed *ad libitum* on live *Artemia sp.* during acclimation and the 6 week experimental period but starved for 24 h prior to sampling (once a week). TBTCI and TPhTCI exposure experiments were performed in 25 litre polyethylene buckets (Carter, *et al.*, 1989) containing silver sand with a nominal TBTCI or TPhTCI concentration of 150 ng g⁻¹ dry weight. To achieve this concentration the method described by Waldock *et al.* (1989) was modified in that TBTCI or TPhTCI in glacial acetic acid was adhered to approximately 20 g of fine deep-sea mud collected from the Porcupine Abyssal Plain, north-east Atlantic (TBT & TPhT concentration < 1 ng g⁻¹) and then mixed into 2 kg of clean sand. Preliminary experiments showed that the concentration of TBTCI and TPhTCI in these preparations decreased to mean values of 121 ng g⁻¹ and 115 ng g⁻¹ dry weight after five weeks, respectively.

Samples for organotin analysis in the subsequent exposure experiments were taken immediately after the addition of TBTCI or TPhTCI and again at the end of the experiment (five weeks) and stored at -20°C. Organotin analysis was performed by GC-FPD according to the method of Waldock *et al.* (1989). Control groups were placed on the same mix of sediments but without the addition of organotin. The water in all buckets was continuously aerated and changed once a week.

The apparent water permeability, drinking and urine production rates of 0-group flounders were determined, enabling an estimation of the net water balance. Measurements were carried out at the beginning of the experiment (t₀) and in weekly intervals for five weeks. The apparent water permeability was determined by measuring the half-time of exchange of tritiated water (THO) as described by Lockwood *et al.* (1973) and adapted for flounders by Hutchinson (1984). The diffusional water flux was calculated from the unidirectional flux (Hutchinson, 1984) and normalised to the fish wet weight (µl g⁻¹ h⁻¹). The drinking rates were determined by placing the animals into a loading medium containing ⁵¹Cr-EDTA and measuring the activity of the imbibed water (Hutchinson & Hawkins, 1990). The urine production was determined from the clearance rate of injected ⁵¹Cr-EDTA from the blood (Babiker, 1975; Hutchinson & Hawkins, 1990) and the net water balance was determined by subtracting the drinking rate from the urine production rate (Smith, 1932).

The osmolality of blood samples and the surrounding medium were measured by the cryoscopic method of Ramsay & Brown (1955). Na^+ -fluxes were measured using $^{22}\text{NaCl}$ (Hutchinson, 1984), modified from Shaw (1959) and normalised to gill area ($\text{nmol mm}^{-2} \text{h}^{-1}$). Gill area was estimated using $y = ax^b$, where y is the total gill area of a given fish [mm^2], x is the fish wet weight [g] and the constants $a = 239.02$ and $b = 0.723$ (Hartl, *et al.* (in press)). At weekly intervals, the fork lengths were recorded to the nearest decimal place with a pair of vernier callipers.

Apart from net water balance data, that first required a square root transformation, all data sets were parametric. Comparisons between TBTCI, TPhTCI and control groups were analysed by a repeated measurement one-way analysis of variance (RM-ANOVA), followed by a Student-Newman-Keuls multiple comparison procedure. Maximum increases or decreases within each treatment group were compared to the respective initial values (t_0) using a paired t -test (Fry, 1993).

Results

Chronic exposure of 0-group flounders to sediment containing 150 ng g^{-1} TBT and TPhT caused a significant reduction of the half-time of exchange ($T_{1/2}$) of

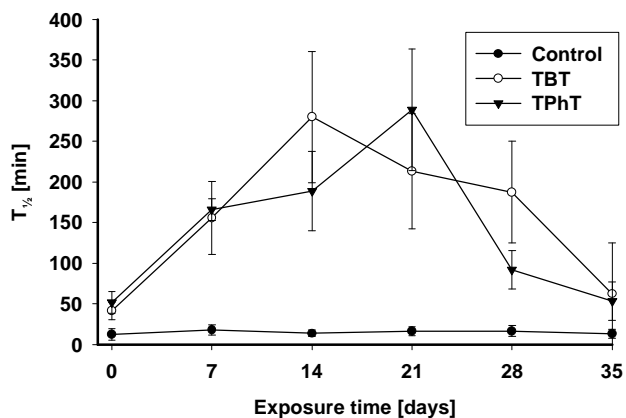


Fig. 1. Halftime of exchange of THO during chronic exposure to 150 ng g^{-1} sediment-associated TBTCI or TPhTCI (minutes; $n = 15 \pm \text{S. D.}$)

tritiated water (THO), compared to that of a control group (Fig. 1). During the first 14 days in the TBTCI group and 21 days in the TPhT group the $T_{1/2}$ had increased from initially 41 to 279 minutes ($P < 0.05$) and 62 to 288 minutes ($P < 0.05$). This in turn was reflected in a decrease in diffusional water flux, falling from 6.19 to 0.58 $\mu\text{l g}^{-1} \text{h}^{-1}$ ($P < 0.05$) in the TBT group and 6.6 to 0.3 $\mu\text{l g}^{-1} \text{h}^{-1}$ ($P < 0.05$) in the TPhT group (Fig. 2).

After two weeks of exposure, $T_{1/2}$ began to decrease steadily, eventually reaching the level that the control group had constantly maintained throughout the experiment. It must be stressed that during most of the experiment, the THO flux across the membranes of both organotin groups was significantly lower than that of the control group ($P < 0.001$). There was no significant difference between the organotin groups. In the TBT and TPhT groups, drinking rates increased in the first three weeks and the first two weeks, respectively, from 0.45 to 0.90 $\mu\text{l g}^{-1} \text{h}^{-1}$ ($P < 0.001$) and from 0.4 to 0.89 $\mu\text{l g}^{-1} \text{h}^{-1}$ ($P < 0.05$) and then towards the end of the experiment, slowly decreased to 0.64 and 0.73 $\mu\text{l g}^{-1} \text{h}^{-1}$ (Fig 3). The drinking rates of the control group did not change significantly ($P > 0.05$). The urine production rate in both organotin groups decreased slightly but was generally

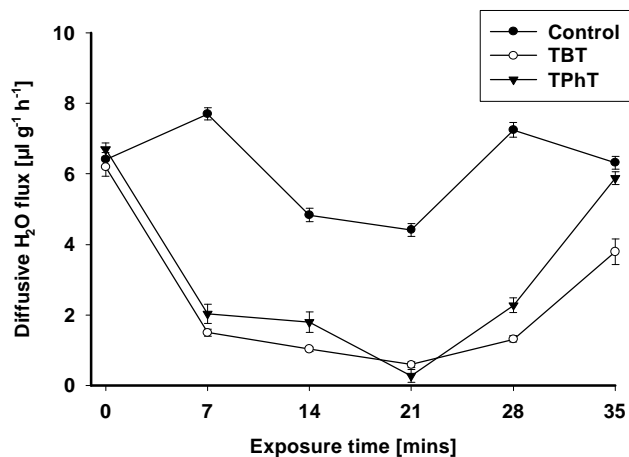


Fig. 2. Diffusive H₂O flux during chronic exposure to 150 ng g⁻¹ sediment-associated organotins ($\mu\text{l g}^{-1} \text{h}^{-1}$; $n = 15 \pm \text{S. D.}$).

never significantly ($P > 0.05$) different from the values at the start of the experiment (Fig. 4). During the first three weeks of the experiment there was no significant difference ($P > 0.05$) between the net water balance of the TBT and control groups. However, during week four the net water balance in the TBT group increased from 0.33 to 0.74 % body weight ($P < 0.001$) and differed significantly ($P < 0.001$) from the control values that were maintained at a stable positive level during the entire experiment.

During the first three weeks the net water balance of the TPhT group peaked at 0.73 % body weight. During week four and five the net water balance returned to the initial value (Fig. 5). Passive Na^+ -efflux rates in the control group remained unchanged during the experiment ($P > 0.05$). In the TBT and TPhT groups, however, passive Na^+ -efflux increased from 32.21 to 88.33 $\text{nmol mm}^{-2} \text{h}^{-1}$ ($P < 0.05$) and 38.1 to 70 $\text{nmol mm}^{-2} \text{h}^{-1}$ ($P < 0.05$), respectively, over the first three weeks of exposure and towards the end of the experiment decreased to 60.3 and 66.6 $\text{nmol mm}^{-2} \text{h}^{-1}$, respectively, and were generally significantly higher ($P < 0.05$) than the values of the control group. (Fig.6).

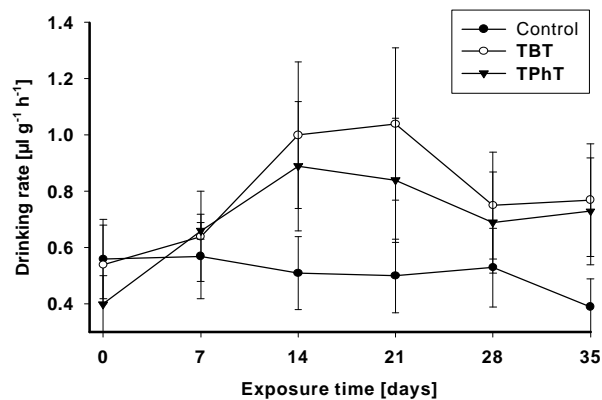


Fig. 3. Drinking rates during chronic exposure to 150 ng g⁻¹ sediment-associated organotins (µl g⁻¹ h⁻¹; n = 15 ± S. D.)

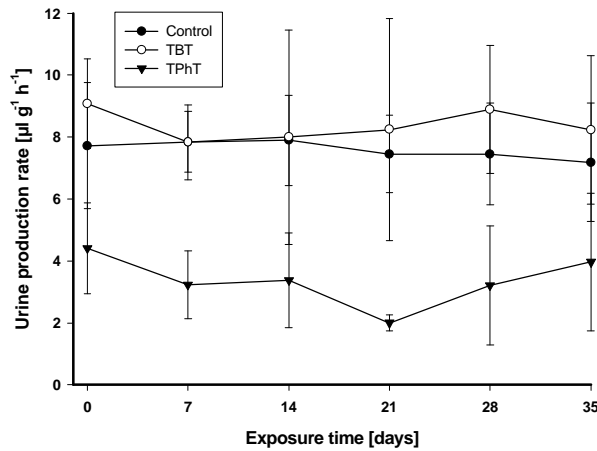


Fig. 4. Rate of urine production during chronic exposure to 150 ng g⁻¹ sediment-associated organotins (µl g⁻¹ h⁻¹; n = 15 ± S. D.).

After Γ groups
 averaged 287 ± 13 and 286 ± 7 mOsmol kg⁻¹ and was significantly lower than the values determined in the control group (309.28 ± 14 mOsmol kg⁻¹; Fig. 7; *P*

< 0.05). The control group showed a 12 % increase in length during the first week and was subsequently reduced and stabilised at 7% per week during the rest of the experiment. While the TPhT group only increased in length by 2 % throughout the experiment, the weekly length increase in the TBT group rose steadily, eventually reaching the control values. During most of the experiment, the increase in length was generally significantly lower (*P* < 0.05) than that of the control group.

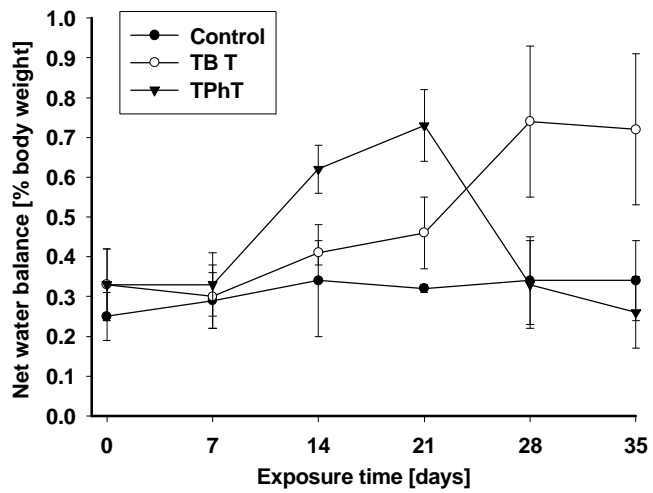


Fig. 5. The net water balance during chronic exposure to 150 ng g^{-1} sediment-associated TBTCI and TPhTCI in % body weight; $n = 15 \pm \text{S. D.}$

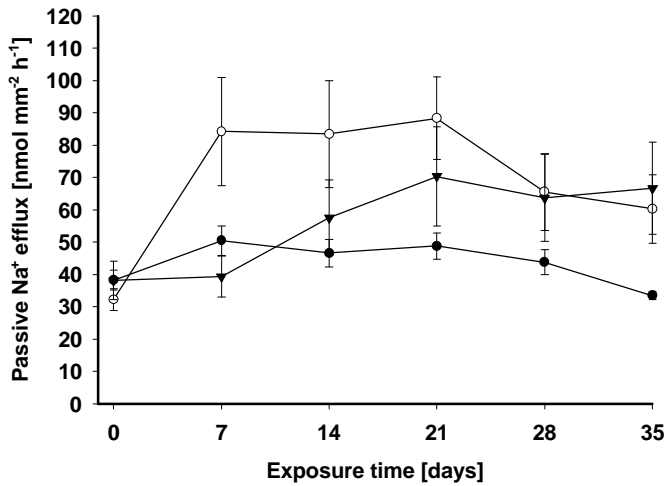


Fig. 6. Passive Na⁺ efflux across the gills during chronic exposure to 150 ng g^{-1} sediment-associated TBTCI and TPhTCI ($\text{nmol mm}^{-2} \text{ h}^{-1}$; $n = 15 \pm \text{S. D.}$).

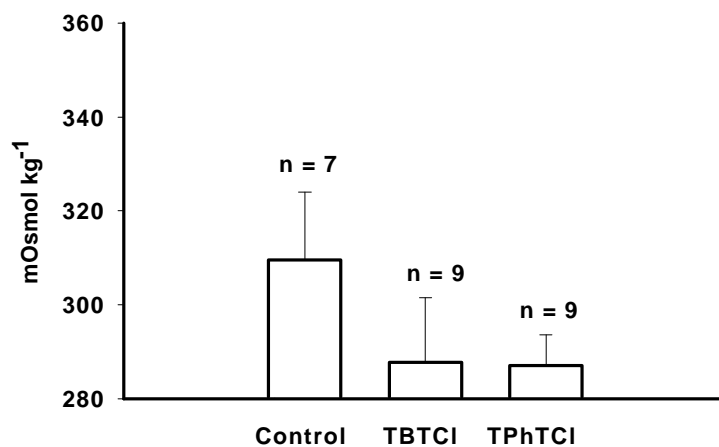


Fig. 7. Blood osmolality after five weeks of chronic exposure to 150 ng g⁻¹ sediment-associated organotin compounds (mOsmol kg⁻¹; mean ± S. D.).

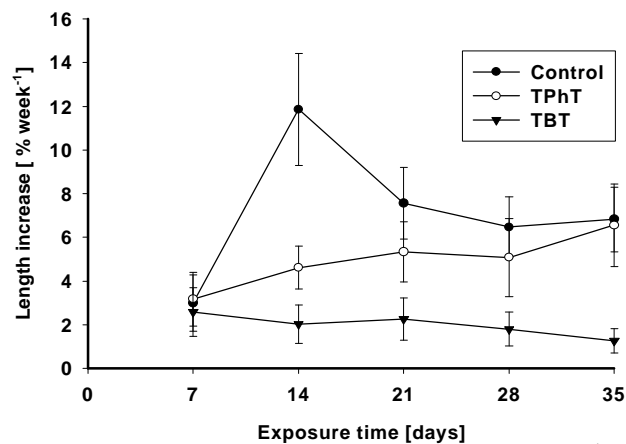


Fig. 8. Length increase during chronic exposure to 150 ng g⁻¹ sediment-associated TBTCI or TPhTCI expressed as % increase week⁻¹; n = 10 ± S. D.).

Discussion

Freshwater adapted euryhaline fish are hyper-osmotic in respect to the external medium. They therefore have not only to compensate for ion loss but also for an osmotic water influx across the gill membrane by adjusting the membrane permeability, drinking and urine production rates (Evans, 1969). The control values in the present study for these measures of osmoregulation are comparable with those reported by Hutchinson & Hawkins (1990). The increased $T_{1/2}$ values in the TBT group (Fig. 1) suggest that TBT interaction with the gills decreased the diffusional flux of THO across the membrane (Fig. 2), indicating a decrease in the apparent membrane permeability.

The effect of organotin compounds on membrane permeability has been widely studied using model membranes. For example, Cullen *et al.* (1997) reported a decrease in membrane 'fluidity', following the addition of TBTCI to the extraliposomal compartment of an egg phosphatidylcholine liposome preparation, that lead to a decreased efflux of encapsulated dimethylarsenic acid by passive diffusion. Heywood *et al.* (1989) recorded changes of membrane structure, such as lysis, caused by tributyltin compounds and suggested that this could lead to an increased permeability. Experiments with fluorescent probes have indicated that TBTCI locates itself in the hydrophobic core of erythrocyte membranes causing haemolysis (Falcioni *et al.*, 1996). They suggested that the oxygen radicals produced during this process could cause structural defects to the membrane by increasing the number of double bonds in the hydrocarbon chains, leading to modifications of membrane permeability.

In all of the above cases model membranes were used in conjunction with organotin concentrations that were several orders of magnitude higher than the sediment concentrations found in the River Itchen, which may explain the observed membrane disruption in those experiments. The gills account for 90 % of the diffusional water flux (Evans, 1969, Motais *et al.*, 1969), so a reduction in gill permeability and subsequent reduction of diffusive water influx will alter water balance, so as to cause an increase in blood osmolality. In an osmoregulator such as *P. flesus* drinking rates and urine production are adjusted in order to offset any elevation of osmolality. A healthy freshwater acclimated flounder would be expected to drink occasionally and to produce large volumes of dilute urine, in order to keep the net water influx and the ion loss at an absolute minimum and therefore the blood osmolality within a narrow range

(Evans, 1979). This behaviour was observed in the control group (Figs. 3 & 4) with the exception of a slight increase of urine production towards the end of the experiment, which may be an artefact caused by handling stress (Eddy, 1981; McDonald & Milligan, 1997). However, Lahlou (1967) found stress-induced diuresis not to be a significant factor in laboratory experiments conducted with *P. flesus*. Seawater-adapted flounder exhibit an increase in drinking rates and a reduction in urine production in order to compensate for increasing blood osmolality. This behaviour was observed in both organotin groups (Figs. 3 & 4) although the fish were kept in freshwater throughout the entire experiment.

This suggests that the fish is compensating for increasing blood osmolality, caused by the reduction of membrane permeability for water. This process can also be observed in freshwater-acclimated fish when subjected to osmotic stress as reported by Lahlou *et al.* (1969) for the goldfish *Carassius auratus*. The data available in the literature suggests that between 62% and 80% of the water swallowed by fish is actually absorbed by the intestine (Smith, 1930; Hickman, 1968; Oide & Utida, 1968; Shehadeh & Gordon, 1969). If this is also true for flounders, then the shift in the osmotic water influx, caused by the enhanced drinking rates, should be reflected by a shift in the net water balance of TBTCI- and TPhTCI-exposed fish as shown in Fig. 5.

A further effect of triorganotins on the gill membranes observed in this study involves the passive efflux of Na^+ , that was significantly increased ($P < 0.05$; Fig 6) and may be contributing further to the changes in blood osmolality observed in the organotin groups (Fig. 7). There would appear to be a metabolic cost attached to the changes produced by exposure to TBTCI and TPhTCI that are manifested as a minimal increase in body length compared to the controls, as shown in Fig. 8. This observation is consistent with the findings of a study by Seinen *et al.* (1981), who observed significant growth retardation and weight loss in rainbow trout yolk sac fry during chronic exposure to 1 ppb TBTCI. Thus the reduced blood osmolality in the organotin groups in this study could be a reflection of the increased osmotic water influx rates caused by stress-induced increase in drinking as a consequence of permeability changes to the gill membranes following the interaction with re-mobilised sediment-associated TBTCI and TPhTCI.

The results presented here also suggest that benthic fish that are in contact with contaminated sediments are more likely to suffer adverse effects to their osmoregulatory system than pelagic species. This suggests further that this

source of exposure may be a more important factor than organotin in the water column, especially as far higher concentrations in water seemed to have little effect on blood osmolality as shown by previous studies (Chliamovitch & Kuhn, 1977; Pinkney *et al.*, 1989).

We conclude from the results presented here that TBTCI and TPhTCI in sediments is capable of significantly disrupting the osmoregulatory functions of an estuarine fish, at concentrations currently found in local sediments.

Acknowledgements

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**CADMIUM TOXIC EFFECTS ON HEART VENTRICLE
OF HALOBATRACHUS DIDACTYLUS–
CHRONIC EXPOSURE STUDY**

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Introduction

Due to its potential as a toxic substance, cadmium is one of the metals to which a special statute in questions of environmental and health is given. As no biological function has been attributed to cadmium, this metal is toxic for the cell, even in low concentrations. Various studies connect cadmium with oxidative stress, since this metal can alter the antioxidant defence system in several tissues of several animals, causing a depletion in the levels of reduced glutathione, as well as an alteration in the activity of antioxidant enzymes, and a change in the structure of the cellular membrane through a process of lipid peroxidation (Jamall et al., 1989; Palace et al., 1993; Sarkar et al., 1995; Zikic et al., 1996). Cadmium has a high potential of toxicity, mainly in the liver and kidney. However, its interaction with the cardiac muscle cell is not well understood. The objective of this work was to analyse antioxidant defence system responses induced by a chronic exposure to a sub-lethal cadmium concentration (1 mg/kg), on heart ventricle of a teleost fish, *Halobatrachus didactylus* (toadfish).

Materials and Methods

H. didactylus individuals were collected from Ria Formosa (South Coast of Portugal) and divided into two groups: Control group (CTRL), injected intraperitoneously (i.p.) at day 0 with 0.9% NaCl, and sacrificed after 6 weeks; Cadmium exposure group (Cd), injected i.p. at day 0 with 1 mg/kg of Cd as CdCl₂ in NaCl 0.9%, and sacrificed after 6 weeks of exposure.

Heart ventricle from each individual was collected after sacrifice and cytosolic and mitochondrial fractions were prepared for determination of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (total GPx and Se-GPx) activities. Lipid peroxidation products were also analysed using TBA method. Cadmium concentrations were determined in total, cytosolic and mitochondrial fractions using flame/furnace atomic absorption spectroscopy.

All parameters studied are presented as averages of measurements taken from 5 individuals in each group. The Mann-Whitney test, the non-parametric equivalent of the analysis of variance between two treatments, was applied to test differences between groups, on all the parameters analysed. The significant level used was 5% ($\alpha=0.05$).

Results and Discussion

The results indicate alterations of the antioxidant defence systems (Table I): SOD seems to be inhibited both in mitochondrial and cytosolic fraction; CAT and GPx activities are not affected significantly after 6 weeks exposure. Subcellular Cd distribution is presented in Table II.

In the experimental conditions described, there was a significant increase in lipid degradation products levels. These results, together with those of the antioxidant enzymes activity, indicate an ineffective response of the cellular defence mechanisms in protecting the cell against the oxidative stress caused by this metal. Lipid peroxidation can be one of the consequences of oxidative stress, a situation that usually occurs when the production of reactive oxygen species (ROS) exceeds that of the antioxidant defence systems. The ROS can be inactivated through the action of antioxidant enzymes or other unspecific antioxidants.

Prior studies in this specie, with the same Cd concentration but in 1 day and 1 week of exposure (Correia et al., 1998), indicate a strong and effective response of the antioxidant enzymes and no significant increase in lipid peroxidation. These results together seem to show that, although antioxidant mechanisms protect the cell during an acute Cd exposure, this protection is not sufficient in longer periods and cellular injury may result.

The process of lipid peroxidation determines the alteration in the structure of cell membrane (Cheeseman, 1993). Future studies are needed to clarify functional consequences of the membrane injury in ventricle tissue.

Table I – Percentual variation of antioxidant enzymes activity and lipid degradation products after 6 weeks Cadmium exposure.

Parameter	Fraction	% variation
		6 weeks
SOD	cytosolic	-22,4*
	mitochondrial	-23,7*
CAT	cytosolic	-10,9
	mitochondrial	8,4
GPx	total	4,6
	Se-GPx	-5,3
Lipid peroxidation	total	36,1*

* significant differences with CTRL groups.

Table II – Subcellular Cadmium distribution ($\mu\text{g/g}$) in the heart ventricle of *Halobatrachus didactylus*.

Fractions	0days	6weeks	
	CTRL	CTRL	Cd
Total	0,341	0,615	5,879
Mitochondrial	0,000	0,235	1,817
Cytosol	0,000	0,044	0,632

Acknowledgement

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**SOCIOECONOMIC ASPECTS AND HEAVY METAL LEVELS
IN FISHES OF THE ORINOCO RIVER
CONSUMED BY CAICARA RIVERINE COMMUNITY,
BOLÍVAR STATE, VENEZUELA.**

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Abstract

The socioeconomic aspects and the metallic levels of Fe, Ca, Na, K and Mg were evaluated in six fish species of the river Orinoco: Caribe (*Pigocentrus cariba*), Morocoto (*Colossoma brachypomun*), Bagre rayao (*Pseudoplatysloma fasciatum*), Curvina (*Plagioscion squamosissimus*), Coporo (*Prochilodus mariae*) and Guaraguara (*Hypostomus* sp.) that are consumed by the residing population in the city of Caicara of the Orinoco, in order to quantify the concentrations of these elements in the fishes' muscular fabrics and to relate them with the preference levels and frequency of consumption of the different social strata in the above mentioned city. The fish samples were captured between the months of November 1998 and March 1999 in the main bed of the river and two of its flood lagoons. The Graffar method was used in a sample universe of 200 families to determine the socioeconomic stratum and the consumption preference for these fish in the population. The socioeconomic study, according to Graffar, indicated that 86 % of population is located in the lower middle class (level IV, 56%) and 28 % in the low class (level V), and these strata have a consumption preference toward the species of more economic

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value. The concentrations of trace metals for all the species oscillated in a range of: iron 19.88 – 47.41; magnesium 958.52 – 4951.32; potassium 11629.41 – 19648.53; sodium 1386.73 – 3566.86; and calcium 387.05 – 2516.32 $\mu\text{g g}^{-1}$ dry weight. The results also indicate that the biggest concentrations of almost all the studied metals are in the species of lesser commercial value. These results allow us to make a recommendation to consume the species of lower economic value, namely Guaraguara (*Hypostomus* sp.), Caribem (*Pigocentrus cariba*) and Coporo (*Prochilodus mariae*), thereby contributing to the achievement of a balanced diet at low cost.

Key words: Orinoco river fish, metallic levels, socioeconomic aspects, fish consumption

Introduction

Caicara city, of the Orinoco riverine community, is the autonomous capital of Cedeño in Bolívar State, representing the largest municipal territory in all of Venezuela (Fig. 1). This city shows the highest potential in the “Eje Orinaco – Apure” Project (a project which will further socioeconomic development in the south-central region of Venezuela). It is located on the east border of the Orinoco River at 66° 10’ 36’’ and 7° 38’ 36’’ north latitude, on a fringe 50 to 100 Km parallel to the river, in the center of Venezuela. The city takes up 66,390 Km² of the 238,000 Km² in Bolívar State, which lies within the 457,095 Km² of the Guayana region. The population of Caicara city has shown accelerated growth at approximately 6.3% annually, leading to present day census of approximately 55,000 persons.

Caicara city’s economy is quite meager, relying mostly on mining production and, to a lesser extent, fishing. The immediate zone of influence is cattle raising, with some agricultural crops emerging. The fishing industry shows great potential but has not yet been properly developed. In light of the growing fishing industry on a national level in recent years, and the fact that increasing prices of red meat have encouraged consumption of other meats, Venezuela will become increasingly dependent on exporting fish and other marine products in the near future (Weibezahn *et al.*, 1990).

The amount of essential metals such as sodium, potassium, calcium, iron, and magnesium found in foods such as meats, vegetables, and especially milk, along with the population's consumption patterns, may serve as a reference for establishing nutritional requirements (Itriago *et al.*, 1997). However, products derived from fresh water fish may also fulfill an important role in this exercise.

It is well known that primary minerals such as sodium, potassium, magnesium, and calcium are necessary substances for living. However, it is lesser known that other metals, such as iron, are also essential to the development, and thus reproduction of living beings. No organic life can develop and survive without the participation of essential metallic ions, including Ca, Fe, Mg, Na and K.

According to Forster and Wittmann (1983) an element is essential when: (1) it can be consistently determined that the element is present in every healthy tissue of living beings, (2) the deficiency symptoms are noted with removal of such elements, which disappear when returned to the tissue, and (3) the deficiency symptoms can be attributed to distinct biochemical defects (above molecular levels).

Venezuela has little information on levels of Ca, Fe, Mg, Na, and K in many foods (Itriago *et al.*, 1997) and in particular freshwater fish. As such, this project includes a study of the presence of these elements in six fish species of the Orinoco River, with the purpose of relating them with socioeconomic factors in the Caicara population, since these elements are of great importance because they exist in many of the physiological processes of living beings.

Materials and Methods

Population of study. A total of 200 families were chosen randomly in the city of Caicara of the Orinoco riverine community, in Bolívar State (Fig. 1). Social strata were assigned via the Graffar method, a standardized method generally accepted worldwide to establish different socioeconomic strata within a population. Through this method, it was determined that 86% of the families occupying this zone are classified in the lower-middle stratum (level IV, 58%) and low stratum (level V, 28%).

Collection and storage of samples. Fifteen (15) examples of each species: Caribe (*Pigocentrus cariba*), Morocoto (*Colossoma brachypomun*), Bagre rayao

(*Pseudoplatysloma fasciatum*), Curvina (*Plagioscion squamosissimus*), Coporo (*Prochilodus mariae*) and Guaraguara (*Hypostomus* sp.) were collected in the principal canal of the river and two of its lakes (Caicarita, Piedrita) between the months of November 1998 and March 1999 (Fig. 1). The fish were captured using trawling nets. The samples corresponding to each location were put separately in plastic bags, ticketed and kept in wine vaults with ice until their arrival at the laboratories (Scott *et al.*, 1999). The muscle tissue samples were taken from above the lateral line, at the beginning of the dorsal fin in each fish, and were subsequently frozen at -15°C until the analyses. All species were identified through taxonomical clues and related bibliographies (Mago – Leccia, 1970; Taphorn and Lilyestrom, 1984; Machado – Allison, 1987a; Mago – Leccia, 1994; Machado *et al.*, 1995).

Analytical methods. The tissue samples were dried in an oven at 80°C at constant, weighed and homogenized. Two grams of dry sample per triplicate were submitted to concentrated nitric acid and analyzed by Atomic absorption of Spectrophotometry with acetylene air flames (Hansen *et al.*, 19990; Malcom *et al.*, 1994; Wood and Van Vleet, 1996) and corrected, using Perkin Elmer 3100 equipment with auto-muscletrader Perkin Elmer AS-51. The calibration patterns and targets were submitted to the same conditions as the samples.

Results and Discussion

The consumption of fishes by the population of Caicara riverine community of the Orinoco and the levels of metals (iron, magnesium, calcium, sodium, and potassium) found in tissue samples in six species of freshwater fish of the Orinoco River: Caribe (*Pigocentrus cariba*), Morocoto (*Colossoma brachypomun*), Bagre rayao (*Pseudoplatysloma fasciatum*), Curvina (*Plagioscion squamosissimus*), Coporo (*Prochilodus mariae*) and Guaraguara (*Hypostomus* sp.) are represented in Table 1 and in Figures 2-7. Similarly, the elements which the human body requires and their respective levels are shown in Table 2, which reveals differences in the concentrations of the 10 elements currently accepted as essential to human life.

Figure 2 presents the consumption preferences of 200 families in Caicara city of the Orinoco, Bolívar State, for nine species of freshwater fish that inhabit the Orinoco river: Caribe (*Pigocentrus cariba*), Morocoto (*Colossoma brachypomun*), Bagre rayao (*Pseudoplatysloma fasciatum*), Curvina

(*Plagioscion squamosissimus*), Coporo (*Prochilodus mariae*), Guaraguara (*Hypostomus* sp.), Pavon (*Chicla ocellaris*), Cachama (*Colossoma macropomun*), and Palometa (*Mylossoma duriventris*). It is noted that consumption is skewed toward species of higher economic value in the regional and national markets, as is the case of the Morocoto, Cachama and Bagre rayao, which show preferences of 27, 15.5 and 19 %, respectively: the lesser percentages for species of lesser economic value, such as the Caribe and the Guaraguara, which received only 4.5 and 0.5 % acceptance. This observation is of utmost significance, because despite the fact that 86% of the population is in the lower social class (level V) and lower-medium (IV), their preference is toward species of high economic value.

However, when we observe and compare the levels of iron, sodium, potassium, magnesium, and calcium in the species of higher and lower economic value (Table 1 and Figures 2-7), it is apparent that concentrations of these elements in fish such as Guaraguara, Coporo and Caribe, which are quoted at more affordable prices, are very similar and in some cases higher than levels found in species of higher economic value. Thus, these species are suitable dietary alternatives of high nutritional value and low cost for strata IV and V within the population.

Figure 3 shows that iron is the metal, of the 5 metals studied, that is found in lower concentrations among the six species studied. These levels are increased in the Morocoto, Guaraguara and Coporo (45.71, 46.74, and 47.71 $\mu\text{g/g}$), which are higher than those in the Bagre rayao and the Caribe by almost a factor of two. The lowest concentrations are found in the Curvina, a fish that is sold at high prices.

Magnesium (Figure 4) is found in highest concentration in the Bagre rayao and the Morocoto (4951.32 and 1557.89 $\mu\text{g/g}$, respectively). However, concentrations in the remaining four species are very similar, all ranging above 900 $\mu\text{g/g}$. These concentrations are above those reported by Barrios *et al.* (1996) for some fruits such as the Lechosa, papaya and the Limonzón, lime (1262 and 665 $\mu\text{g/g}$, respectively).

The comparative results of concentrations of calcium and sodium for this study are presented in Figures 5-6. Calcium and sodium concentrations range between 1000-3500 $\mu\text{g/g}$, with the highest levels in the Morocoto (sodium 35,566.86; calcium 3,200.72 $\mu\text{g/g}$) and the Guaraguara (sodium 2,944.86; calcium 2,406.41

µg/g). This draws attention to the low calcium levels compared to the remaining species, which may be a function of physiological aspects of the fish, which may not be able to retain large quantities of calcium in its tissue compared to the other species in the study.

Figure 7 reports potassium levels. This element was the one that was most abundant in the six species studied. Concentrations of this element ranged between 10,000-20,000 µg/g, with the highest levels in the Morocoto (16,868.1µg/g) and Guaraguara (1,961.53 µg/g), and the lowest levels in the bagre (11,629.41µg/g). The diversity of metal concentrations for any species in particular, compared to the other species, may be influenced initially by factors such as the following: place of capture, dietary habits, and physiological activity.

It is important to note that nutritional metal levels of the species of low economic value, such as the Guaraguara, Coporo and Caribe, are comparable with those of high economic value. As such, there are no true correlations between purchasing power in the population of Caicara of the Orinoco riverine community and the nutritional levels of species of high or low economic value. This lack of correlations is due to a lack of research on this topic. This study can serve as the cornerstone for further developmental work to educate the population on the nutritional and economic factors related to the different freshwater fish species, particularly in light of the precarious economic situation facing this region.

Conclusions

The 86 % of families in Caicara city of the Orinoco River, Bolívar State, belong to strata IV and V, and are inclined to consume species of higher economic value such as the Morocoto, Bagre rayo and Cachama. However, such species can be substituted by other species of lesser economic value, such as the Guaraguara, which have comparable levels of metals Fe, Mg, Na, K and Ca.

There is no true correlation between nutritional value, purchasing power in the Caicara population of the Orinoco riverine community, and levels of essential metals Fe, Mg, Na, K and Ca in the freshwater fish species, including the Caribe (*Pigocentrus cariba*), Morocoto (*Colossoma brachypomun*), Bagre rayao

(*Pseudoplatysloma fasciatum*), Curvina (*Plagioscion squamosissimus*), Coporo (*Prochilodus mariae*) and Guaraguara (*Hypostomus* sp.).

Therefore, we can recommend consumption of species of lesser economic value, such as the Guaraguara, Caribe and Coporo, thereby contributing to the achievement of a balanced diet at low cost.

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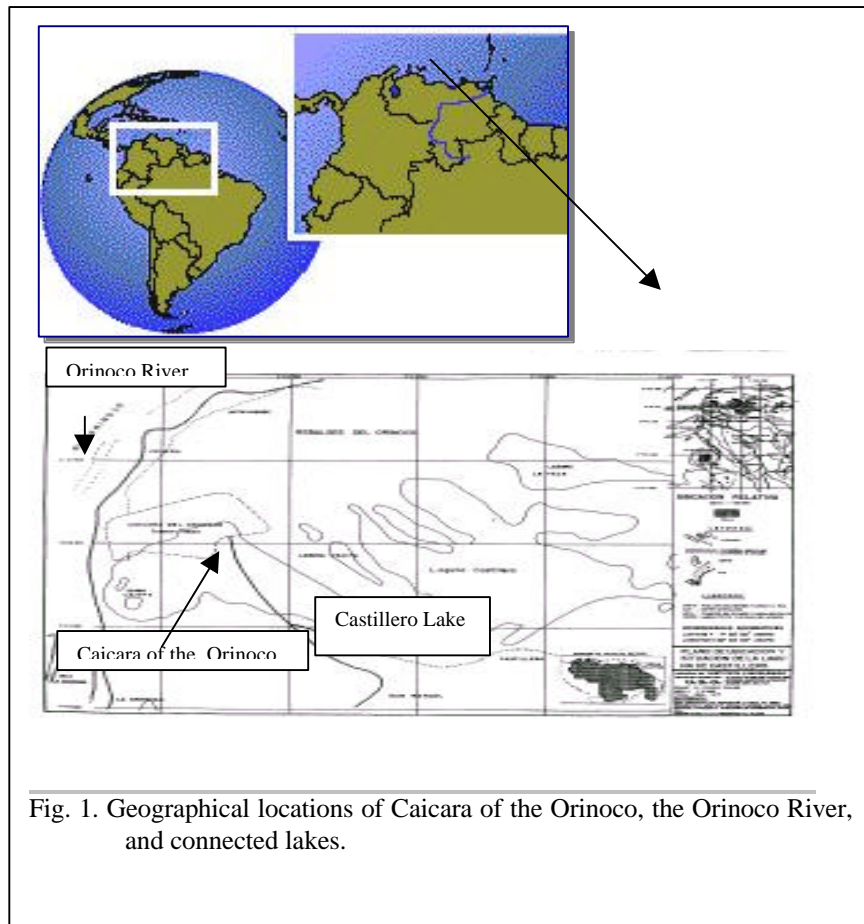
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Table 1. Average (\pm SD) concentrations ($\mu\text{g/g}$) of iron (Fe), magnesium (Mg), potassium (K), sodium (Na) and calcium in muscular tissues in six fish species of the Orinoco River (n=15).

Species	Fe	Mg	K	Na	Ca
<i>Caribe</i> (Pigocentrus cariba)	26.24 \pm 3.60	982.25 \pm 236.70	14125.90 \pm 679.04	2368.39 \pm 132.35	1668.65 \pm 125.44
<i>Coporo</i> (Prochilodus mariae)	47.41 \pm 11.01	1039.20 \pm 73.86	13412.42 \pm 597.82	2255.24 \pm 305.44	2516.32 \pm 361.16
<i>Curvina</i> (Plagioscion squamosissimus)	19.88 \pm 9.28	958.52 \pm 166,80	13179.67 \pm 978.36	2095.78 \pm 367.13	2392.82 \pm 105.12
<i>Morocoto</i> (Colossoma brachypomun)	45.71 \pm 7.63	1557.89 \pm 303.64	16868.10 \pm 724.23	3566.86 \pm 784.63	3200.72 \pm 411.12
<i>Bagre rayao</i> (Pseudoplatysloma fasciatum)	26.03 \pm 5.08	951 \pm 236.04	11626.41 \pm 365.23	1386.73 \pm 47.39	387.05 \pm 33..38
<i>Guaraguara</i> (Hypostomus sp.)	46.74 \pm 8.03	1242 \pm 170.24	19648.53 \pm 427.48	2944.86 \pm 419.52	2406.41 \pm 177.81

Table 2. Essential metals and their respective concentrations in humans (expressed in mg/70 kg of body weight (Forstner & Wittmann, 1983).

<i>Metal</i>	<i>Fe</i>	<i>Mg</i>	<i>K</i>	<i>Na</i>	<i>Ca</i>	<i>Mn</i>	<i>Cu</i>	<i>Zn</i>	<i>Co</i>	<i>Mo</i>
<i>mg/70 kg body weight</i>	7.000	4.000	250.000	70.000	1.700.000	30	150	3.000	1	5



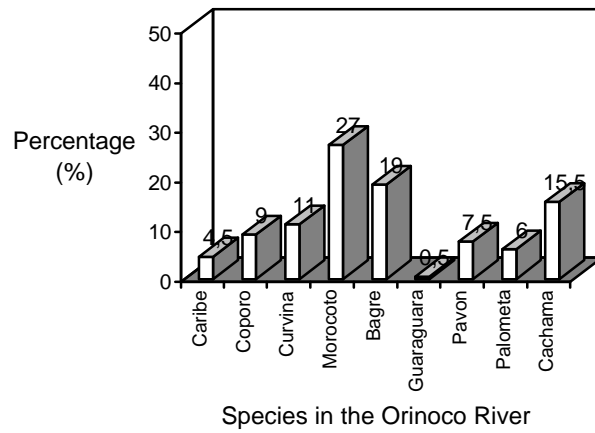


Fig. 2. Consumption (%) of freshwater fishes in the Caicara riverine population of the Orinoco River, Bolívar State, Venezuela.

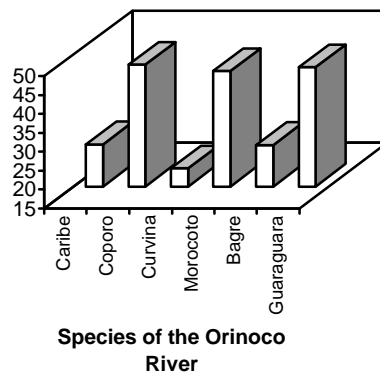


Fig. 3. Comparison of iron levels (µg/g) in muscle tissues of six fish species in the Orinoco River.

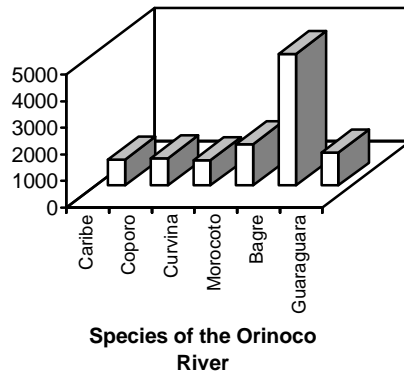


Fig. 4. Comparison of magnesium levels ($\mu\text{g/g}$) in muscle tissues of six fish species in the Orinoco River.

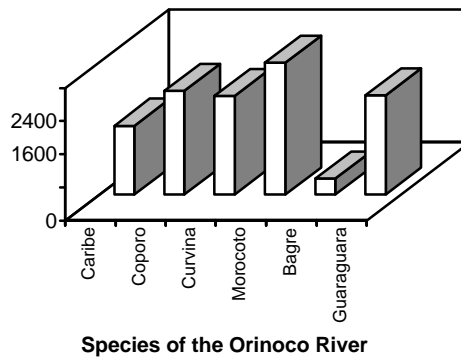


Fig. 5. Comparison of calcium levels ($\mu\text{g/g}$) in muscle tissues of six fish species in the Orinoco River.

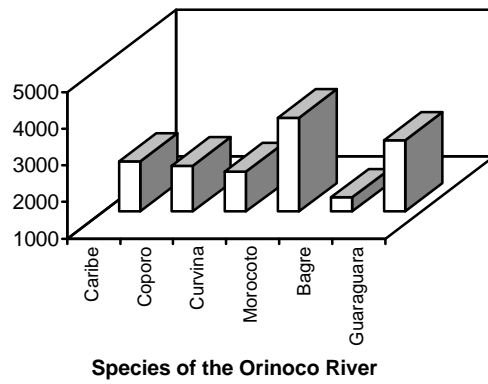


Fig. 6. Comparison of sodium levels ($\mu\text{g/g}$) in muscle tissues of six fish species in the Orinoco River.

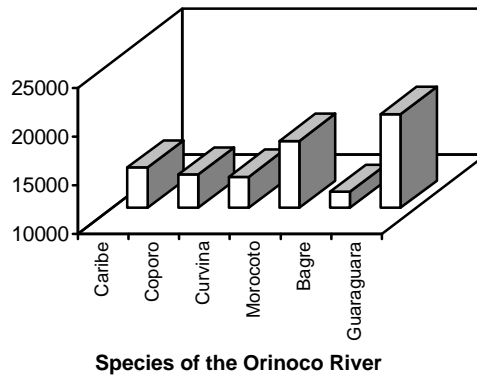


Fig. 7. Comparison of potassium levels ($\mu\text{g/g}$) in muscle tissues of six fish species in the Orinoco River.

SKELETAL MUSCLE ULTRASTRUCTURAL PATHOLOGY
IN THE TROPICAL FISH *Collossoma macropomum* (Cuvier, 1818)
TREATED WITH A HERBICIDE

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Abstract

Chemical drugs used for plague control produce skeletal muscle alterations manifested as a myopathy, this has been reported for DDT. In Venezuela, the control of aquatic weeds in fish cultivation ponds, is performed with herbicides such as 2-chloro-4,6-bis-ethylamine-s-triazine. This lead us to undertake the study of skeletal muscle ultrastructure of the tropical fish *Collossoma macropomum* (Characiformes: Characidae) in order to determine possible changes in muscle fibre ultrastructure. Samples from the epiaxial

muscle of three juvenile specimens were processed by conventional techniques for Transmission Electron Microscopy and observed in a Hitachi H-7100 electron microscope operated at an accelerating voltage of 75 kV. Muscle fibre changes consisted of swelling of sarcofibrillar elements, the presence myelin-like figures and autophagic vacuoles with mitochondrial debris. Contractile system hypercontraction and sarcomere disorganization, nuclear envelope and mitochondrial swelling was also observed. Electron dense granules were located in close relation with mitochondria in subsarcolemmal spaces. Additionally, alterations in motor nerve and end-plates were observed. The findings we observed have been reported as common myopathic changes in other vertebrates due to herbicide toxicity.

Keywords: *Colossoma macropomum*, skeletal muscle alterations, Transmission Electron Microscopy, herbicide

Introduction

The need of control of the aquatic weeds on fish cultivation ponds is high as for other reservoirs of water, as for human consumption. For that reason many chemical products of varied properties are broadly used (Maroto, 1989), specially when the invaded areas are very extensive (Huet, 1978; Barret, 1988). The application of chemical compounds is a great responsibility because even non-lethal doses can cause sub-clinical alterations (Medina and Urbina, 1992), then it is necessary to know the effect of those compounds on the ultrastructure of organisms such as fishes, because the presence of possible severe damage could explain changes appeared in its behaviour like the loss of balance. The purpose of the present work is to determine the effect of the chemical compound 2-chloro-4,6-bis-ethylamine-s-triazine in the ultrastructure of the skeletal muscle of the cachama (*Colossoma macropomum*), a very important and widespread species and for pisciculture in our country; we have a good knowledge on its feeding, and very especially reproduction (Goulding, 1980; Cervigon, 1983; Mago, 1970). It is well-known that fish has a high muscular demand for the locomotion system in order to maintain the balance in swimming (Bone, 1978). When the skeletal muscle is altered the lost of balance may occur, as was reported by Andrade et al., (1982). The observation that the use of the chemical compound 2-chloro-4,6-bis-ethylamine-s-triazine in cachama cultivation ponds provokes changes in the balance and behaviour motivated the study of the possible morphological changes underlying the mentioned behaviour in this species.

Materials and Methods

The epiaxial musculature of three juvenil specimens of *Colossoma macropomum* (Characiformes: Characidae) were used, specimens came from the cultivations of the Experimental Station Guanapito of the National Fund for Agricultural Investigations (FONAIAP), Guárico State, Venezuela and treated with 2-chloro-4,6-bis-ethylamine-s-tryazine 2.5 ppm concentration of active ingredient, for 72 hours. Were carried out muscle samples of approximately 2 mm diameter, were fixed with glutaraldehyde 2,5% and OsO₄ 1%. In both was used Millonig phosphate buffer (320 mOsm and pH 7.4). The dehydration was achieved in

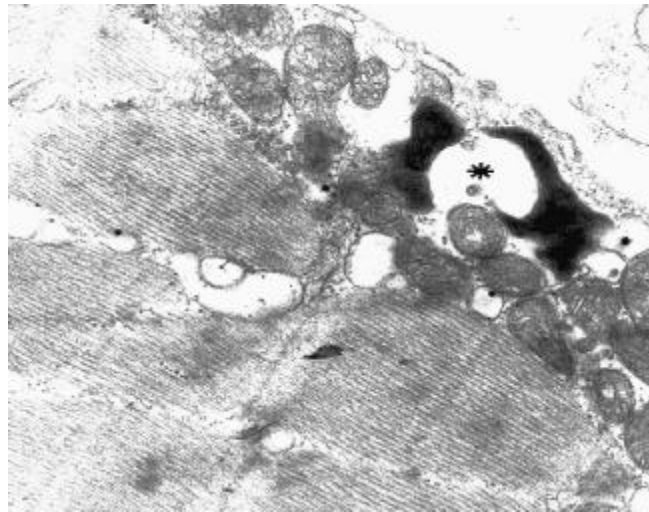


Figure.1. Observe the autophagic vacuoles formed from mitochondrial debris in the subsarcolemmal space (*) Magnification: X 30,000.

increasing ethanol concentrations, then infiltration with propylene oxide and a mixture of propylene oxide-epon (1: 1), and inclusion in the resin LX-112 (Ladd Research, Inc. Burlington) for 48 hours at 60 °C. 60 - 90 nm sections were

obtained with a Porter-Blum MT2-B ultramicrotome, contrasted with uranyl acetate and lead citrate and observed in Hitachi H-7100 transmission electron microscope at an accelerating voltage of 75 kV.

Results

Ultrastructural observation of *Colossoma macropomum* treated with the herbicide showed normal mitochondrial and autophagic vacuoles with mitochondria debris in the subsarcolemmal space (figure 1). Additionally, myelin-like figures were

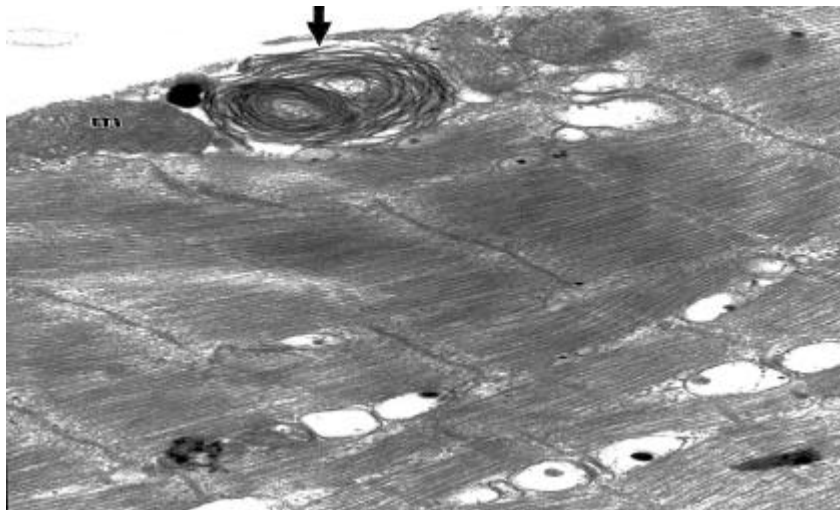


Figure 2. - Observe a mitochondrion (m) and the presence of a myelin-like figure. Magnification: X 60,000 .

also seen (figure 2). Autophagic vacuoles with mitochondrial debris were also found next to the nucleus (figure 3). Electron dense granules were observed

in the subsarcolemmal space next to mitochondria. Muscle fibre atrophy with increased intermyofibrillar and subsarcolemmal space was a frequent observation

(figure 4). Sarcotubular system elements were swollen and apparently fragmented (figures 5 and 6) next to these elements areas of hipercontraction were seen (figure 5). Motor end-plate terminal axon exhibited mitochondrial

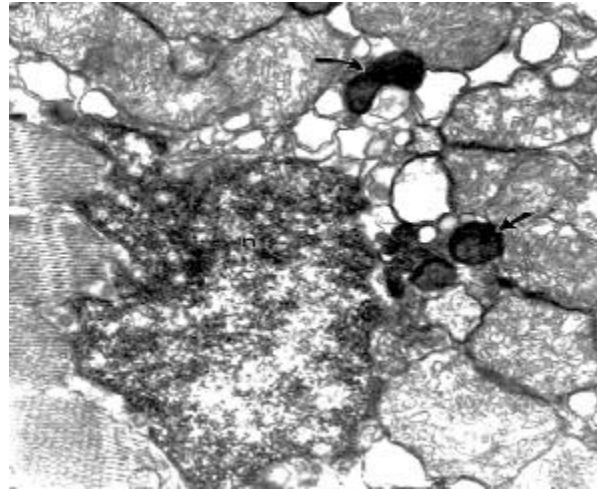


Figure 3. Observe autophagic vacuoles (arrows) next to the nucleus (n). Magnification: X 24,000.

alterations and a decreased synaptic vesicle number (figure 7). Also the motor nerve showed axon and myelin alterations (figures 6, 7 and 8).

Discussion

The herbicide 2-chloro-4,6-bis-ethylamine-s-triazine with no doubt produces favorable results in the fight against aquatic weeds. Nevertheless, special attention should be have in its use by the damage that can cause to the fishfauna. Although fish tolerates doses used for the control of the overgrowths in the bodies of water (Medina and Urbina, 1992), very small dose, in comparison with those tolerated and recommended by the makers can cause damages in skeletal muscle ultrastructure, as it is shown in this investigation,

where we could observe the pathological effect caused by the herbicide 2-chloro-4,6-bis-ethylamine-s-triazine in the fish *Colossoma macropomum*, treated with a dose of 2.5 ppm, which is smaller in 50% than the maximum dose recommended by the makers and higher in approximately 50% that the recommended minimum dose whose values oscillate between 1.6 ppm and 3.3 ppm (Avila et al., 1995).

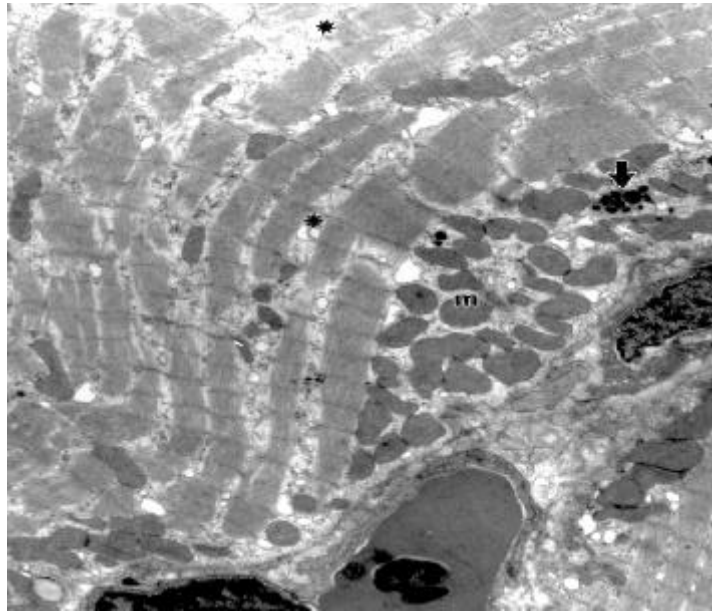
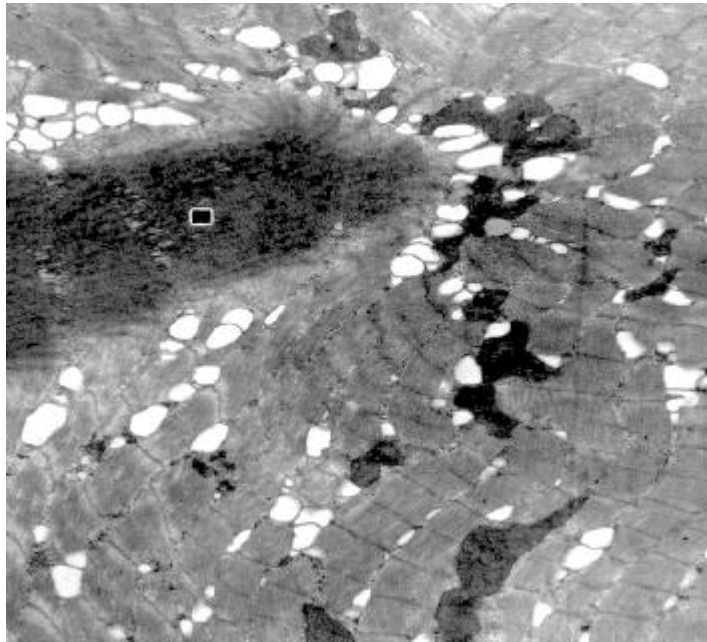


Figure 4 -. Note dense granules (arrow), mitochondria (m) and increased intermyofibrillar spaces (asterisk). Magnification: X 12,000.

Figure 5 -. Note hipercontraction area (square) and swolled elements of the sarcotubular system. Magnification: X 15,000.



In the herbicide 2-chloro-4,6-bis-ethylamine-s-triazine, the form of action of its triazine is very similar to the one of those derived from urea, and they are used, therefore, in emergency treatments, against the submerged grasses, as Gianni and Diani suggest (1971).

In spite of the fact that a given herbicide dose is tolerated by fish, it causes alteration that produce loss of balance and speed in swimming (Medina and Urbina, 1992); which may be related to skeletal muscle fibre alterations, such as atrophy, sarcomeric disorganization and fragmentation of sarcotubular system elements as was shown in the present investigation. Particularly in this context were findings of hypercontraction, changes in motor nerve and end-plates. These changes are compatible with a neurogenic atrophy. However, the

possibility is not excluded that effects of the herbicide are also carried on directly on muscle fibres.

There is a scarcity in the literature not only in Venezuela, but also at international level in relation to ultrastructural aspects of possible muscle damage use for comparison in connection with herbicide toxicity, although several works have been published on normal fish skeletal muscle ultrastructure (Bergman, 1967; Kilarski, 1967; Korneliusse and Nicolaysen, 1973; Akster, 1981). However it is known that some herbicides and insecticides can cause clinical pathologies as DDT (Huet, 1978). Application of Kepone[®], chemical compound used as insecticide, it was shown the inhibition of Na⁺ K⁺-ATPase in cell plasmalemma, increasing membrane permeability with cell swelling and organelle alterations of gill pavement mucous cells (Mallat et al., 1995). Additionally, the compound Chloramine-T produces epithelium swelling and hypertrophy of lamellar epithelial cells (Powell et al., 1995). Mentioned findings could represent a support in relation to our suggestion that 2-chloro-4,6-bis-ethylamine-s-triazine affects skeletal muscle ultrastructure of *Colossoma macropomum* provoking changes in its swimming behaviour.

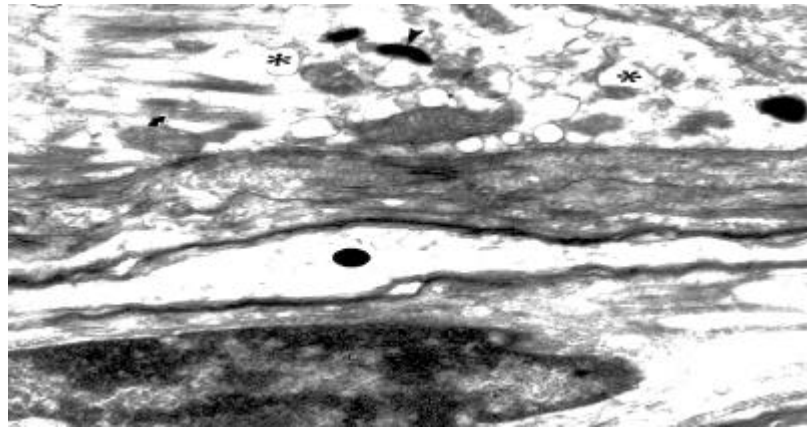


Figure 6 -. Note dense granules (arrow-head), fragments of sarcotubular system elements (asterisk) and disorganized myofibrils (arrow). The motor nerve axon also appeared altered (circle). Magnification: X 34,000 .

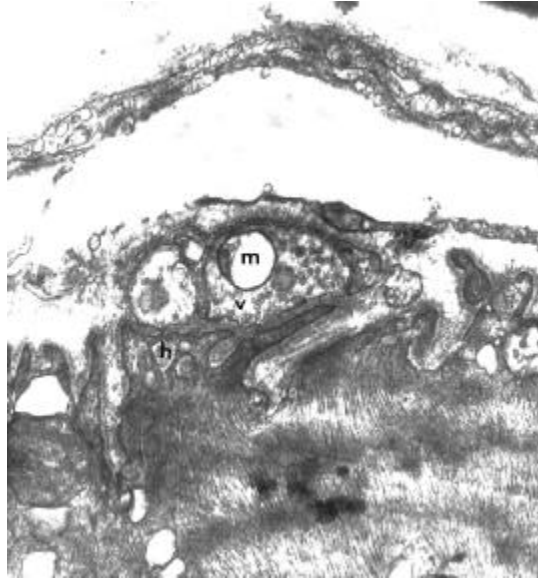


Figure 7-. Note in motor end-plate terminal axon on altered mitochondrion (m) scarce synaptic vesicles (v) observe irregular secondary synaptic clefts (h). X 51,000.

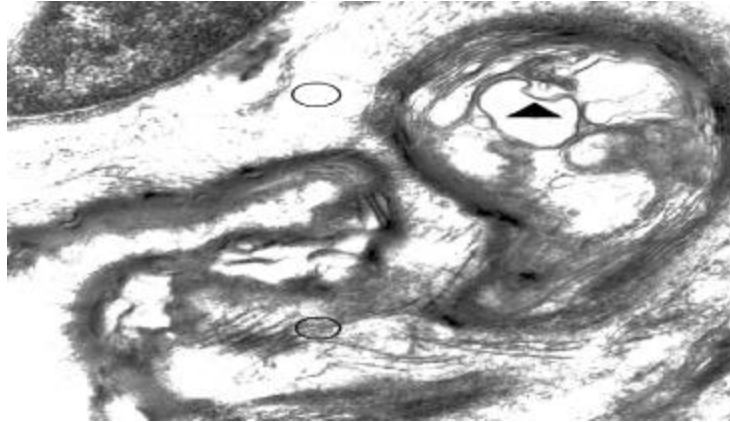


Figure 8.- Observe altered motor nerve and axon (triangle) and the increased collagen fibrils (circles). Magnification: X 51,000.

Conclusions

- 1.-The herbicide 2-chloro-4,6-bis-ethylamine-s-triazine produces alterations of skeletal muscle fibre ultrastructure and motor innervation.
- 2.- Muscle fibre alterations are found in contractile and sarcotubular systems and in mitochondria.
- 3.- Changes of motor innervation were observed in motor nerve and endplates.
- 4.- Our findings could explain loss of balance and swimming behaviour of *Collossoma macropomum* affected by the herbicide 2-chloro-4,6-bis-ethylamine-s-triazine.

Acknowledgement

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METABOLIC EFFECTS OF THE PESTICIDE METHYL PARATHION

(FOLIDOL 600[®]) ON *Brycon cephalus* (matrinxã), A TELEOST FISH.

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Introduction

The poisoning by pesticides from agricultural fields is a serious water pollution problem and its environmental long-term effect may result in the incidence of poisoning of fish and other aquatic life forms. (Jyothi and Narayan, 1999).

Folidol 600[®] is a widely used commercial product, which contains methyl parathion as the active substance. It is heavily employed in agricultural fields as well as in fish culture tanks to kill aquatic larva of insects. It usually causes different kinds of environmental contamination and accidents concerning humans and native fauna. (Silva et al., 1993).

The knowledge of sublethal effects of xenobiotic compounds on hematological parameters, enzyme activities and metabolite concentrations is very important to delineate the health fish status and provide a future understanding of ecological impacts. These pesticides act by causing inhibition of cholinesterase enzymes (ChE) by formation of enzyme inhibitor complex (O'Brien, 1976) and damaging the nervous system. These effects may result in metabolic disorders.

Associated to cholinesterase activities, a study of other enzymes such as phosphatases and aminotransferases closed to intermediary metabolite determination provides a wider view of metabolism. Therefore, the aim of this work was the determination of the effects of different Folidol 600[®] concentrations on *B. cephalus* metabolism.

Materials and Methods

Animals and treatments

B. cephalus, a native tropical fish of the Amazon region, weighing about 270g, were collected from CEPTA-IBAMA, Pirassununga (SP) – Brazil and acclimated to laboratory conditions for two weeks. During the acclimation period, fish were maintained in tanks of 2,000 liters with continuous water flow, artificial aeration under controlled temperature (25 - 27°C), natural light cycle and fed *ad libitum* with a commercial feed. Fish were not fed 12 h prior to the experiment to prevent organic matter in the water.

Experimental design

After acclimation, fish were divided in groups and transferred to the laboratory into six glass aquaria (six fish per aquarium) and maintained during 24h to recovery of handling stress with continuous water flow and controlled temperature. One aquarium was used as control, where six fish were maintained for 4h in a static environment, with constant aeration. The other five fish groups were exposed to 0.5, 1.0, 2.0, 5.0 and 7.0 ppm of Folidol 600[®] for 4h in a static environment with the same aeration conditions. After 4h of exposure to pesticide, blood samples were collected from caudal vein, the fish were killed and the tissues were excised.

Blood samples

A blood sample was used for hematological parameters determination. Hematocrit (HTC) was determined by microhematocrit centrifugation technique. The red blood cell counting (RBC) was done in a Newbauer chamber and the hemoglobin concentration was measured spectrophotometrically by Drabkin's method. Another sample of blood was centrifuged at 12,000g for five minutes and the plasma was used for enzymatic assays and metabolites were determined after suitable dilution.

Tissues samples

Liver and muscle samples were removed and quickly chilled in liquid nitrogen and stored at - 20°C for subsequent analysis of enzymes and metabolites. Prior to the assays the tissues were properly homogeneized and centrifuged at 5° C.

Enzyme assays

Alkaline and acid phosphatase activities were quantified in liver and plasma, by modification of Bretauiere and Spilman, (1983) and Moss, (1983) and Bergnoyer and Beach, (1983) colorimetric method, using p-nitrophenil phosphate as substrate. Liver was used to give final assay concentration of 1,5 mg/ml of buffer. Plasma was used without dilution.

Plasma aminotransferase activities of alanine (ALAT) and aspartate (ASAT) were quantified by a modification of Reitman and Frankel's method (1957).

Acetyl (AChE), butyryl (BChE) and propionylcholinesterase (PChE) activities were quantified by modification of Ellman et al. (1961), using acethylthiocholine, butyrylthiolcholine and propionylthiocholine as substrate respectively and 5,5'-dithiobis – 2 nitrobenzoic acid (DTNB) as the chromogen. These activities were measured in plasma centrifuged at 14,000 rpm for 20 minutes.

Metabolic intermediaries

Lactate (Harrower and Brown, 1972), piruvate (Lu, 1939) and glucose (Buboie et al., 1956) were quantified in plasma and muscle of *B. cephalus*. For this analysis, tissues were homogeneized in perchloric acid 0.6N and centrifuged at 3,000 rpm for 1 minute, to give a final concentration of 5µl/ml and 5mg/ml respectively. The liver and muscle glycogen was measured as reported in Bidinotto et al. (1998).

Statistics

Enzymatic activities, metabolic intermediates and hematological data were analyzed by Mann-Whitney Test and the accepted level of confidence was 5%. (Zar, 1984).

Results

Concerning the hematological parameters, it was observed that fish exposed to lower concentrations of Folidol exhibits increase of HTC values and higher haemoglobin percentage. The group exposed to higher concentrations of Folidol shown different responses (table1).

Table1. Hematologicals parameters from *B. cephalus* exposed to Folidol 600[®].

Treatments	Hematologicals parameters		
	RBC x 10 ⁶ mm ⁻³	HTC %	Hemoglobin%
Control	37.50 ± 2.65	33.2 ± 1.47	4.13 ± 0.22
0.5ppm	36.08 ± 1.12	38.3 ± 1.68 *	5.47 ± 0.39 *
1.0ppm	35.90 ± 2.11	41.4 ± 1.29 *	4.50 ± 0.60
2.0ppm	30.90 ± 1.25 *	34.5 ± 1.21	4.96 ± 0.33
5.0ppm	28.90 ± 1.21 *	30.7 ± 1.22	5.18 ± 0.10 *
7.0ppm	31.90 ± 1.29 *	39.6 ± 3.34	5.42 ± 0.21 *

High levels of plasma inhibition for cholinesterases were observed in *B. cephalus* exposed to Folidol (table 2). At lower concentration (0.5ppm) it was observed 50 – 54% of inhibition of these enzymes, ranging 90% as fish were exposed to 5.0 and 7.0ppm of pesticide.

Table 2. Values for plasma activity of cholinesterases of *B. cephalus* exposed to Folidol 600[®].

Treatments	Cholinesterase activities (mU/ml)		
	AChE	PChE	BChE
Control	67.5 ± 6.41	26.5 ± 3.99	6.1 ± 0.85
0.5ppm	36.9 ± 8.84*	14.4 ± 3.30*	3.1 ± 0.55 *
1.0ppm	13.7 ± 5.08*	5.64 ± 1.48*	2.08 ± 0.31*
2.0ppm	16.5 ± 3.33*	6.73 ± 0.93*	2.6 ± 0.16*
5.0ppm	7.2 ± 1.36*	3.49 ± 0.6*	1.47 ± 0.3*
7.0ppm	6.7 ± 1.74*	2.8 ± 1.05*	0.66 ± 0.1*

The values are means \pm S.E.

* Values statistically significant compared to control values at $P < 0.05$.

The plasma aminotransferase activities were decreased for fish exposed to higher Folidol concentrations (fig.1).

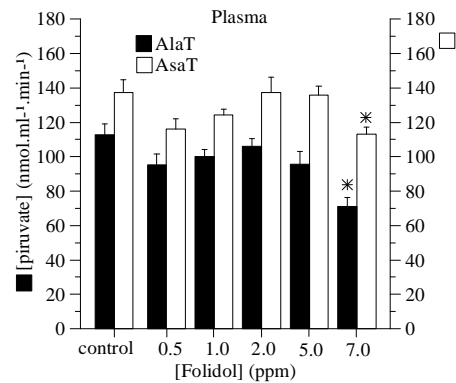


Figure1. AlaT and AsaT plasma activities of *B. cephalus* for different Folidol concentrations.

It was observed that there was an increase of alkaline and acid phosphatases on plasma and liver of matrinxã exposed to higher Folidol concentrations (fig.2 and fig.3). The white muscle and plasma glucose contents did not change in fish groups exposed to Folidol concentrations (fig.4).

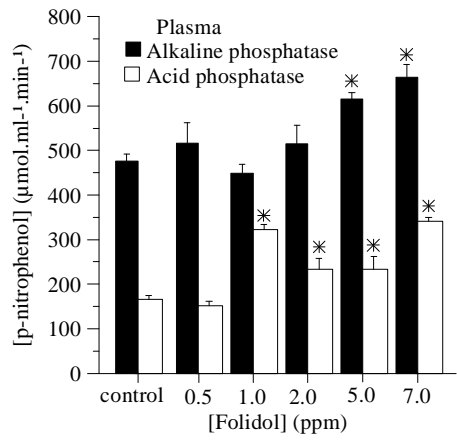


Figure 2. Alkaline and acid phosphatases activities in plasma of *B. cephalus* in different Folidol concentrations.

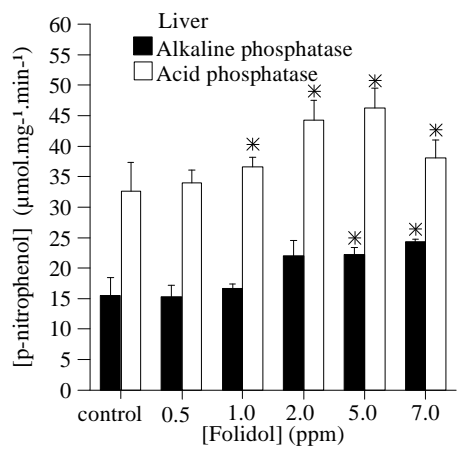


Figure 3. Liver alkaline and acid phosphatase activities of *B. cephalus* in different Folidol concentrations.

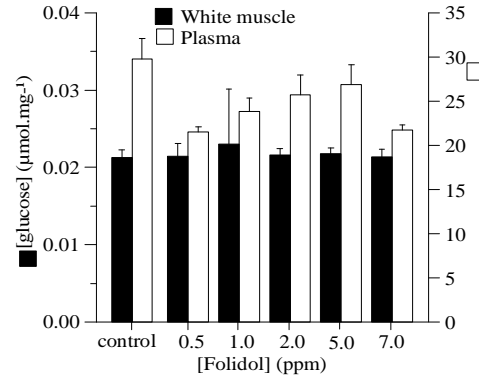


Figure 4. Plasma and white muscle glucose contents of *B. cephalus* in different Folidol concentrations.

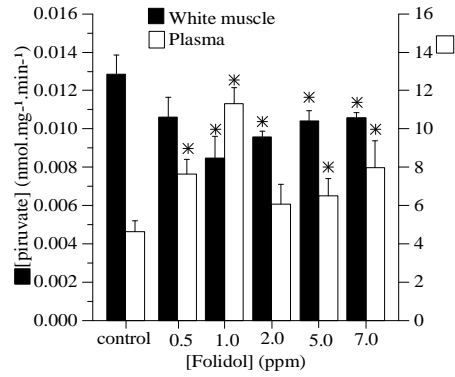


Figure 5. Plasma and white muscle pyruvate contents in *B. cephalus* for different Folidol concentrations.

The plasma pyruvate contents were higher in the groups exposed to Folidol as compared with control group but the white muscle showed opposite metabolic behavior (fig.5).

The white muscle lactate values were significantly higher for all exposed groups as compared with the control, but there were not differences in plasma lactate contents between the control and the exposed groups (fig.6).

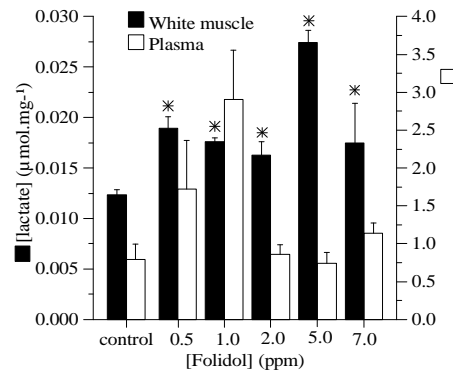


Figure 6. Plasma and white muscle lactate contents of *B. cephalus* in different Folidol concentrations.

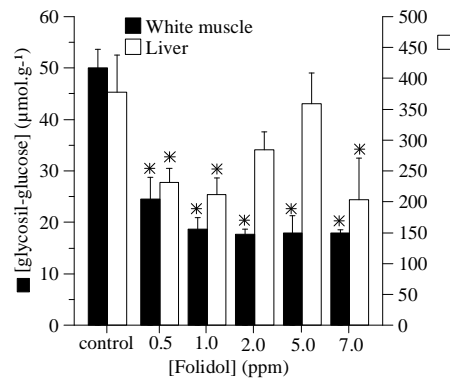


Figure 7. Liver and white muscle glycogen contents in *B. cephalus* for different Folidol concentrations.

The liver and white muscle bulk of *B. cephalus* glycogen exposed to Folidol were significantly lower than control values (fig.7).

Discussion

Our results show that the fish group exposed to the lower concentration of Folidol increases the HTC and haemoglobin contents without increase of RBC. This increase of haematological parameters is classically showed by fish submitted to organophosphate pollution (Natarajan, 1984 and Lal *et al.*, 1986 apud Heath, 1995). On the basis of such results we can suggest that 4 hours exposure to low pesticide concentration (0.5 – 1.0ppm) leads the fish to usual haematological response from pesticide stress. According to Areechon and Plumb, (1990) and Heath, (1995) this response probably occurs due to damage to gill tissues, producing internal hypoxia and stimulation of erythropoiesis. However, the fish groups exposed to higher concentrations of pesticide developed responses probably resulting from the lack for keeping haematological parameters, probably due to exhaustion or damage of haematopoietic tissue.

Effect on Cholinesterases

A significant inhibition level of ChE was detected for *B. cephalus* exposed to Folidol. The AChE, PChE and BChE activities of *B. cephalus* were inhibited by 50 – 54% in 0,5ppm group exposed, reaching 90% of inhibition in 5ppm group exposed, which reveal the high methyl parathion toxicity for the specie. Higher activity of AChE than BChE and PChE was observed due to the substrate specificity (Frobert *et al.*, 1997).

According to Silva Filho *et al.*, (1999), plasma AChE of *P. mesopotamicus* (115g) exhibited high inhibition (73, 88 and 90% for AChE, PChE and BChE respectively) when exposed to 0.2ppm of methyl parathion for 4 hours. These high values of inhibition for *P. mesopotamicus* compared to *B. cephalus* may be due to differences of weight, rate of uptake, detoxification and activation of the pesticide in the body.

The treatments containing the higher insecticide concentration showed very high inhibition of enzymes, however in the same order of the lower concentration treatments. Strauss and Chambers, (1995) suggest, in this case, that AChE saturation may be reached with the lower concentrations of pesticide and the fish should have some mechanisms for disposing of or adapting to higher exposure levels.

Effects on enzyme activities

The decrease of aminotransferases may be attributed to liver damage. This response has been reported for *Carassius auratus* exposed to lead poisoning (Fantin et al., 1988). The increase of plasma and liver acid phosphatase may be associated either with the decrease in stability of liver lysosome membranes or with tissue damage. This enzyme is associated with lysosomal activity. Gill *et al.*, (1992) speculate that acid phosphatase elevation reflects proliferation of lysosomes in attempt to sequester the toxic xenobiotic.

The increase of alkaline phosphatase was observed in *B. cephalus*. Such result is observed as a consequence of osteoblastic activity increase or due to intra and extra hepatic obstructions of biliary passage (Jyothi and Narayan, 1999).

Effects on intermediary metabolites

It was observed a decrease of liver and muscle glycogen in some treated groups and unchanged concentrations of white muscle and plasma glucose. That means a glycogen mobilization, probably to maintain the glucose level and the glycolysis, as usually observed for other fish exposed to a variety of sub-lethal concentrations of organophosphorous compounds (Rany et al., 1990; Gill *et al.*, 1990, 1991). The pyruvate decreases and the lactate contents increases in white muscle. These responses suggest a fermentative strategy of matrix when submitted to methyl parathion.

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**EFFECTS OF ETHYNYLOESTRADIOL
ON TURBOT (*SCOPHTHALMUS MAXIMUS*) LARVAE.**

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

Endocrine disruption in fish has been a popular focal point in recent years. Research has (so far) focussed mainly on identifying what substances contaminating aquatic environments have endocrine disrupting qualities. Furthermore, such research has been conducted primarily on adult freshwater fish species. Very little is known about how these substances affect marine fish, in particular their early life stages.

17 α -ethynylestradiol (EE₂) is a synthetic oestrogen, a common component of the contraceptive pill. It is normally excreted, in harmless conjugated form, into many waterways. However, research has shown that EE₂ conjugates found in sewage effluent can be deconjugated or rapidly degraded, thus restoring the potential of EE₂ to disrupt fish endocrine systems (Larsson *et al.*, 1999). EE₂ has been found to be a potent xeno-oestrogen in many fish species, mimicking the effects of natural oestrogens.

Purdom *et al.* (1994) tested the effects of EE₂ on immature rainbow trout (*Onchorynchus mykiss*) after finding hermaphroditic fish in the lagoons of some sewage treatment works. They found that exposure to EE₂ could induce vitellogenesis, a process normally restricted to adult female fish at times of sexual maturity. EE₂ exposure was also found to induce vitellogenin production in channel catfish (*Ictalurus punctatus*) (Nimrod & Benson, 1996). Injections of EE₂ was found to cause phenotypic sex reversal of genetic males to females in medaka (*Oryzias latipes*) (Papoulias *et al.*, 2000). Nash *et al.* (1997) and Kime & Nash (1999) found that parental exposure of zebra danios (*Danio rerio*) to 5ng/L EE₂ prior to spawning caused cytostatic disruption of embryonic development of eggs during the early gastrula period.

The aim of this investigation was to assess the effects of different concentrations of EE₂ on turbot (*Scophthalmus maximus*) larvae (a marine species) after being exposed to the synthetic oestrogen over a 72-hour period from 2 days post-hatch. Mortality rate, yolk sac volume, larval length and larval morphology were analysed.

Materials and Methods

50 1-day-old turbot larvae (fertilised embryos purchased from the Isle of Man, UK) were placed in 12 moulded glass tanks, each containing 350 ml seawater with antibiotics. The tanks were kept at a constant temperature (15°C) and light intensity.

The larvae were allowed to acclimatise to the tanks for 24 hours before the tanks were inoculated with one of 4 different concentrations of EE₂: 1000 µg l⁻¹, 500 µg l⁻¹, 250 µg l⁻¹ and 100 µg l⁻¹. Two control tanks were also set up: an ethanol (solvent) control tank (1000 µg l⁻¹ ethanol) and a seawater-only control tank. Two replicate tanks were assigned to each treatment.

Approximately 270 ml of seawater from each tank was replaced each day with fresh, aerated, filtered seawater, and the test tanks were re-inoculated with the nominal concentrations of EE₂ (the ethanol (solvent) control tanks were also re-inoculated daily). Any dead larvae in the tanks were counted and removed with a pipette.

The larvae were exposed to EE₂ for a 72-hour period. At the 24, 48 and 72-hour intervals, dead larvae were counted and removed and live larvae were sampled (for future analysis).

Mortality rate, larval length, yolk sac volume and larval morphology were analysed.

Larval length and yolk sac volume differences between treatments were compared using the Kruskal-Wallis test.

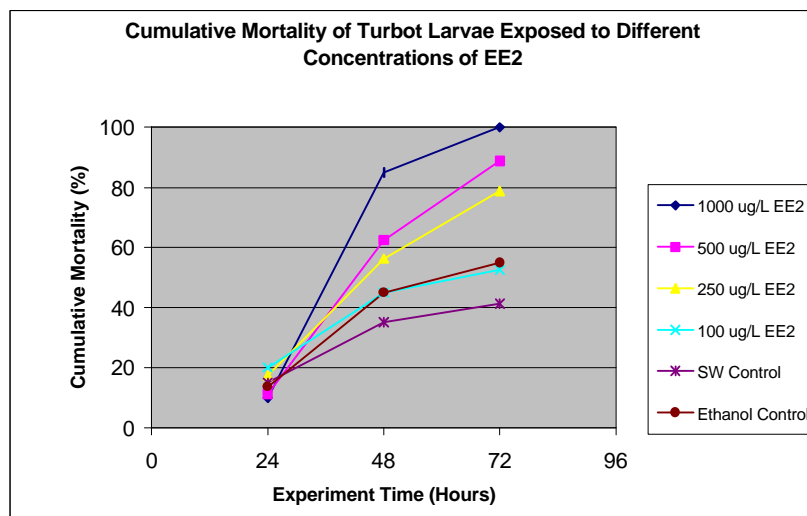


Figure 1. Cumulative Mortality

Results

Results show a marked increase in larval mortality at EE₂ concentration above 250 µg l⁻¹ (Figure 1).

Statistical analysis showed no significant difference in total larvae length (from tip of mouth to tip of tail fin) of larvae from the 6 different treatments over the 24-, 48- and 72-hour periods (Figure 2).

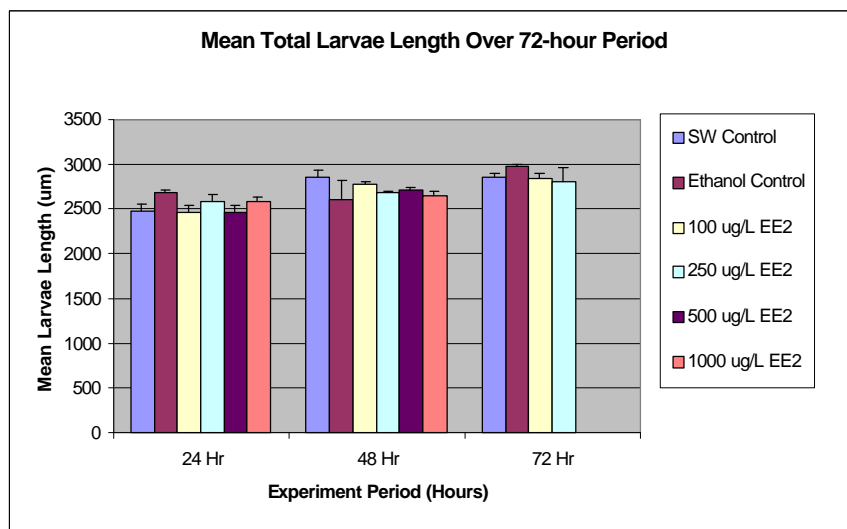


Figure 2. Total Larvae Length Over The Experiment Period.

Differences in larval yolk sac volumes between treatments were also found to be statistically non-significant.

Conclusions

Although EE₂ concentrations above 250 µg l⁻¹ were found to be lethal to larvae, the synthetic oestrogen (at any of the four concentrations) was found not to have an effect on larval morphology. This indicates that at certain concentrations, EE₂ appears to induce a physiological response rather than a morphological response. Future experiments will therefore, concentrate on the mode of toxicity of EE₂ to the early life stages of marine fish.

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KIDNEY ULTRASTRUCTURAL ALTERATIONS
IN THE TROPICAL FISH *Colossoma macropomum* (Cuvier, 1818)
TREATED WITH HERBICIDE

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Abstract

It is known that herbicides used for weed control may affect behaviour in cultured fish ponds. However, no morphological observations have been provided to explain it. Because of that, we decided to undertake the present research to study possible ultrastructural alterations in the kidneys of the tropical fish, *Colossoma macropomum* (Caraciformes: Caracidae). Samples from

juvenile specimens were processed according to routine techniques for Transmission Electron Microscopy and observed in a Hitachi H-7100 electron microscope operated at 75 kV. Alterations seen in kidney tubules included loss of plasmalemma and cell interdigitations, misshaped mitochondria, decrease in rough endoplasmic reticulum and free polysomes and the presence of autophagic vacuoles and primary lysosomes. These results could be associated with the role of the kidney in the control of hydromineral metabolism explaining fish behaviour in terms of kidney tubules pathology.

Keywords. *Colossoma macropomum*, kidney tubules, Transmission Electron Microscopy, herbicide

Introduction

Toxic effects of biochemical and physiological systems are ultimately expressed as changes in cellular and subcellular morphology. Thus, histopathological change is an integration of molecular lesions (Anderson, 1990). With the use of selected transmission electron microscopy (TEM), questions concerning specific alterations at the cellular level may be answered by analysis of relative amounts and conditions of organelles within affected cells (Hinton & Lauren, 1990).

The posterior kidney of freshwater fishes is largely dedicated to the production of copious dilute urine and it has little responsibility for ion or acid-base balance. In marine fish, where they are opposite osmotic gradients, urine flow is severely reduced by elimination of all but the proximal tubules. In some marine species, water loss is further reduced by elimination of glomerular filtration altogether and renal function depends solely on tubular secretion. The kidney of the fish receives the vast majority of postbranchial blood, and because of that, we can expect renal lesions in the fish when toxicant agents exist in the environment. Therefore, a study of these possible kidney changes may be expected to be a good indicator of environmental pollution.

In recent years the use of chemical agents such as pesticides and herbicides to control the plague of weeds has increased the environmental pollution spoiling the ichthiofauna living in culture and the rivers containing them.

The aim of this research is to study the effect of an herbicide on the kidney of *Collossoma macropomun* (Pisces, Characeae), a species widely found in South America from the Orinoco basin to the Amazon basin river. In Venezuela, it is abundant in the Guanare, Portuguesa, Meta, Apure, Caroní and Orinoco rivers. Also, they have a high aquaculture potential because they can be cultured and reproduced in captivity (González y Heredia, 1998).

Materials and Methods

Collossoma macropomun was exposed during 72 hours to sublethal doses of the herbicide 2-chloro-4,6-bis-ethylamine-5-triazine (2.5 ppm). This concentration was taken as the mean value between the extremes recommended for the industry (1.6 to 3.3 ppm). The average lethal concentration for this species is 62 ppm (Medina, 1995). After that, fishes were decapitated and the kidney was rapidly dissected, fixed with glutaraldehyde (2.5%) in Millonig buffer (pH = 7.8, 320 mOsm) for 45 min, trimmed to give blocks of approximately 1 mm³ which were washed three times with Millonig buffer (pH = 7.8) for 1 min. These blocks were postfixed for 1 hour in osmium tetroxide (1%) in the same buffer for 1 hour at 4°C, washed for 15 min in distilled water and dehydrated gradually through an ascending concentration of ethanol at 4°C for 5 min in each stage. They were submerged twice in propylene oxide (15 min at room temperature) and infiltrated with a 1:1 mixture of propylene oxide-resine for 30 min. and four changes of pure resin (LX-112) in each stage. Finally, they were placed in plastic moulds for 48 hours at 60 °C. Sections of 80 nm were cut on an ultramicrotome Porter-Blum MT2-B, transferred to copper grids (200 mesh), stained with uranyl acetate and lead citrate and observed in a Hitachi H-7100 electron microscope at 75 kV.

Results

The kidney of *Collossoma macropomum* is usually a fused organ lying in a retroperitoneal location just ventral to the spinal column and often intermeshed with its processes. The herbicide 2-chloro-4,6-bis-ethylamine-5-triazine, is commonly used in weed control in ponds of cultured fishes. In 2.5 ppm concentrations, it affected the kidney of animals studied in the present research. Different alterations were observed at the ultrastructural level, including vacuolation of cytoplasm of epithelial cells from convoluted proximal tubules and increased autofagic vacuoles (Fig. 1). The apical border of these cells

exhibited numerous microvilli, vesicles and vacuoles (Fig. 2 and 3). Mentioned tubules contained some cells with the basal region showing abundant mitochondria (Fig. 4) as in the control (Fig. 5) but with numerous vesicles and vacuoles not seen in the normal condition. Other cells presented less abundant mitochondria in the interdigitations. In some cases, endothelial cell cytoplasm looked clearly altered (Fig. 6), showing the area nuclei near the basal border (Fig. 7). Capillaries next to this border contained autophagic vacuoles and irregularly located pinocytotic vesicles (Fig. 8). Autophagic vacuoles were also observed in glomerular capillaries (Fig. 9).

Discussion

The effect of stress is evident at several levels of biological organization in teleost fishes. The kidney of these fishes proved to be a sensitive indicator of environmental pollution. Fewer renal histological studies have been conducted with organic pollutants than with metals. Wester & Canton (1986) exposed medaka to an isomer of the insecticide Lindane (i.e. β -hexachlorocyclohexane) and examined several tissues. They found prominent glomerular hyalosis to be an indicator of renal toxicity, but the more interesting observation was that of an apparent estrogen-like activity that resulted in hermaphroditism in males and vitellogenesis in both sexes after three months of exposure. Several commercial antibiotics also produce renal lesions. Lauren *et al.* (1989) found tubular degeneration and eosinophilic, proteinaceous, intratubular casts and hyaline droplets, and an increase in the amount of hemosiderin or melanin like intertubular deposits in rainbow trout fed with the antibiotic fumagilin. Hyaline droplet formation results from tubular reabsorption of plasma protein lost to the urine by glomerular damage. Intratubular casts are markers of damage to the tubule cells themselves. Intramuscular injection of gentamycin sulphate resulted in thickening and sloughing of the glomerular epithelium in channel catfish *Ictalurus punctatus* (Rolf *et al.*, 1986). Coho salmon, *Oncorhynchus kisutch* treated with intraperitoneal injections of tobramycin also showed epithelial necrosis, sloughing of the epithelium, and the accumulation of necrotic debris within the tubule lumen.

In the last three years pesticide studies have been carried out on the effect that these chemical compounds produce on reproductive functions through the endocrine system. It is known that atrazine, 2,4-d-metribuzin and mancozelo produce endocrine disruption (Struger & Painter, 1997). Wester *et al.* (1990) in a study carried out on medaka and guppy, and Thomas (1990) on rainbow trout

exposed to toxicants, found that proximal tubules produced misshapen mitochondria and an increased number of lysosomes. These results agree with those obtained in this research. We also found misshapen mitochondria and an increased vacuole number with large diameters. The use of doses in the lower level (1.6 ppm) of 2-chloro-4,6-bis-ethylamine-5-triazine can be recommended to control weeds, thus avoiding irreversible damage to fish, because we observed some alterations in the kidney. Possibly, at this level, the fish can compensate some of these alterations. It is necessary to continue the study of this effect.

Acute death in cells without somatic death may lead to a series of important reactions for the recovery of an organic tissue. These types of changes may also serve as biological markers of environmental stress effects. When the magnitude of the stressor is enough to cause cellular lesions but not the death of the organism, changes may be observed with light or electron microscopy. Quantitative changes within specific organelles of affected cells are often responsible for the cellular lesions characteristic of chronic or adaptive cellular lesions. These changes include degeneration (cellular swelling), accumulation of cytoplasmic inclusions, and changes in cell and nuclear volumes. Accumulation of triglycerides in the cytoplasm of affected cells is a common indicator of acute, subacute and chronic activity. Triglycerides accumulate in vacuoles within the cytosol and may occupy large portions of cytoplasm. Powell *et al.* (1994) used Chloramine T as a prophylactic and therapeutic agent in freshwater aquaculture ponds. These morphological changes are consistent with a compensatory mechanism for the remedial uptake of ions, suggesting that chloramine T increased epithelial ion permeability coincident with a possible influx of water leading to intercellular edema. Chloride cell proliferation and intercellular edema may also have affected gas exchange across the branchial epithelium (Powell *et al.*, 1994). Also, another organochlorine insecticide, Kepone (chlordecone) altered mitochondrial volume by the compensatory effect (Mallat, 1994).

From our results, it is possible to conclude that pathological renal changes in *Colossoma macropomum* can be used as markers of environmental pollution and monitored by transmission electron microscopy (TEM).

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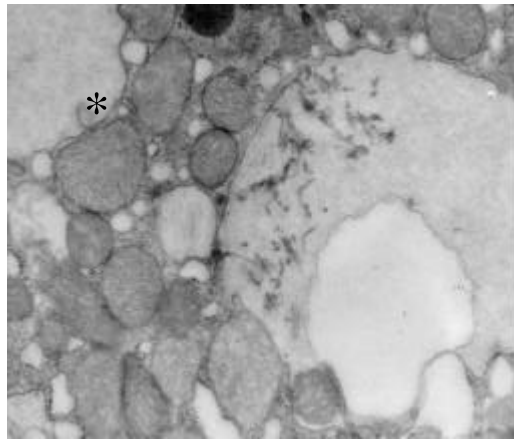


Fig. 1. Electron micrograph showing autophagic vacuoles (*) and vesicles (arrows). X 24,000.

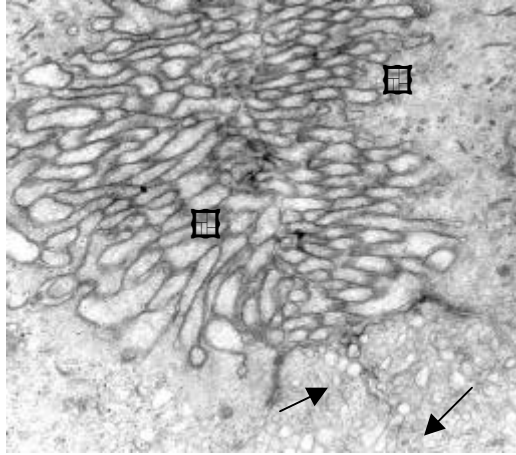


Fig. 2. Note microvilli (square) and vesicles (arrows). X 36,000.

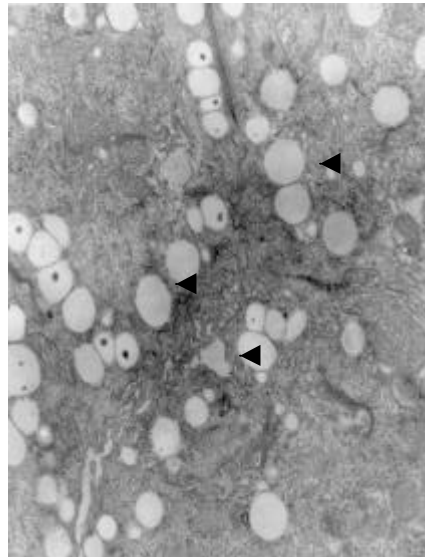


Fig. 3. Electron micrograph of kidney tubules of *Colossoma macropomun*. Observe numerous vacuoles (arrowheads). X 24,000

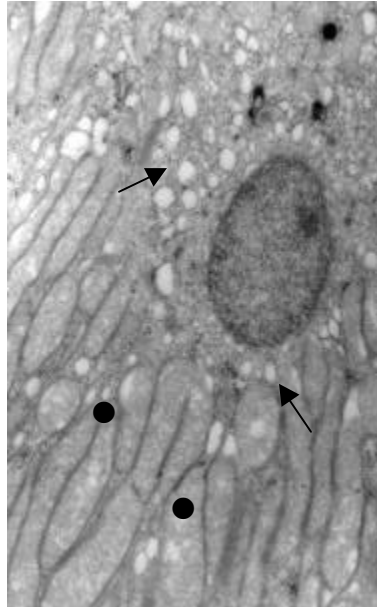
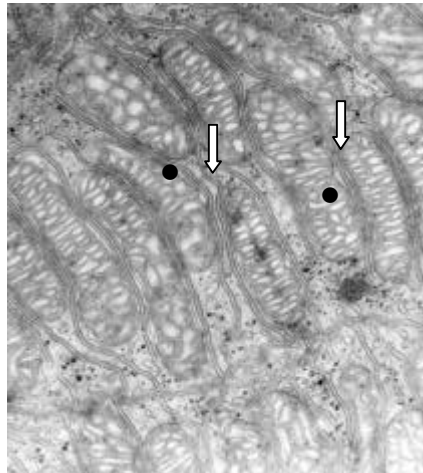
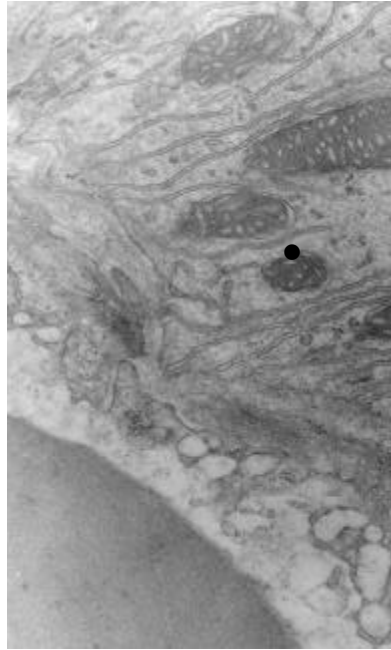


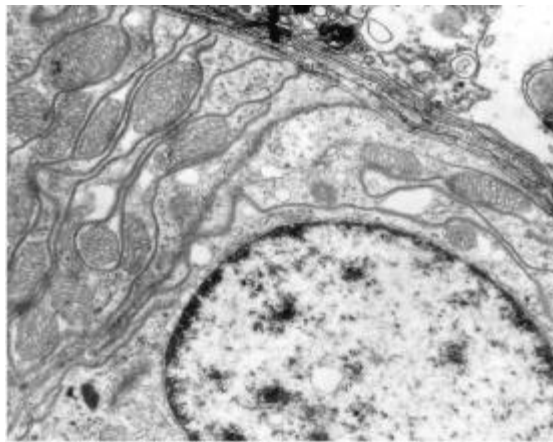
Fig. 4. It is possible to see abundant mitochondria (circles), vesicles and vacuoles (arrows). X 12,000.

Fig. 5. Mitochondria (circles) are surrounded by interdigitations (white arrows). X 36,000.





Mitochondria (circles) are not covered completely by endothelial cell cytoplasm. X 36,000.



The nucleus (N) next to a few interdigitations. X 18,000.

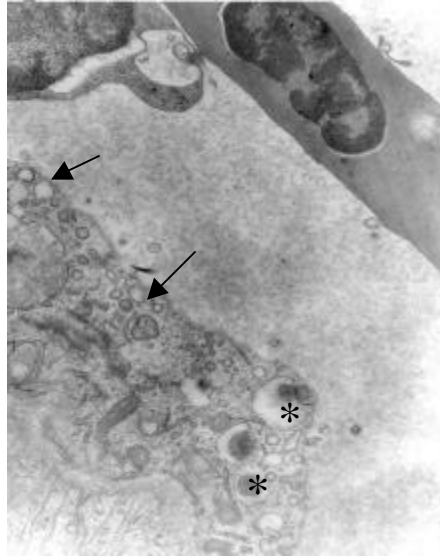


Fig. 8. Autophagic vacuoles (*) and vesicles (arrows) in the endothelial cell cytoplasm. X 30,000.

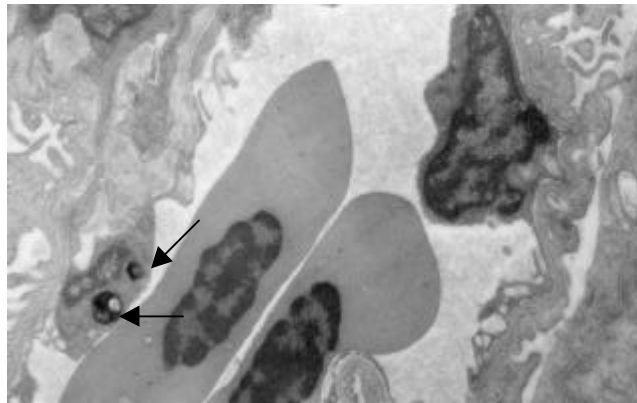


Fig. 9. Note autophagic vacuoles (arrows) in the glomerular capillary endothelial cell cytoplasm. X 15,000.

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**SEX DIFFERENTIATION IN SONGSARI,
ORYZIAS LATIPES EXPOSED TO BISPHENOL A**

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Abstract

The effects of bisphenol A(BPA) on gonadal sex differentiation and maturation in *Oryzias latipes* were investigated. Fish were exposed to aqueous solutions of BPA at nominal concentrations of 50, 100 and 200 µg/l from 2 days to 70 days of age. In process of sex differentiation, advanced oocyte development was observed when compared to the testicular growth. Ovaries were composed of the oocyte of the chromatin-nucleolus stage and peri-nucleolus stage in 20 days after hatching. Otherwise testes contained a number of the spermatogonia and spermatocytes in 30 days after hatching. In the process of sex differentiation, gonadal development was not different in the controls and BPA treatment groups until 30 days after hatching. In contrast, 70 days after hatching advanced development of oocytes in the ovary was observed from BPA treatment groups when compared to the controls, and inhibition of development of spermatogenesis in the testis was observed from BPA treatment groups when compared to the controls. In sex ratio of songsari, more females than males were identified in the BPA 50 and 100 µg/l treatments in comparison to the controls

and BPA 200 µg/l treatment. The range of BPA effects was dependent on the sex of the songsari, and the concentration of the BPA in the water.

Introduction

An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function (European Commission, 1996). And during the past several years, the environmental endocrine disruptors have been deal with the sensitive concern in scientific, popular and political field. Bisphenol A (BPA) is widely used as the primary product of poly-carbonated plastic and epoxy-wax. Poly-carbonate is at liberty to use as the drink pac and earthen vessel and epoxy-wax apply a primary product to the coating of can, bottle and water pipe (Brotons et al., 1995). Evaluation of reproductive organ development in the male offspring of female wistar rats exposed to BPA in the drinking water has been reported (Diomond et al., 1998). Occurrence of ovo-testis (hermaphroditism) was observed in medaka, *O. latipes* exposed to β-hexachlorocyclohexane and p-nonyphenol (Wester and Canton, 1986; Gray and Metcalfe, 1997). In the present study we histologically investigated on the sex differentiation in songsari exposed from hatched larvae to up to 70 days post-hatch to BPA.

Methods and Materials

Chemicals

The Bisphenol A ethoxylaten (BPA) used in all experiments was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, USA. A stock solution of 100 mg/ml was prepared by dissolving BPA in acetone.

Fish

Songsari used in this study were from a breeding stock that has been maintained at marine research institute of Cheju national university for over 3 months. Fish were maintained in recirculating Cheju Island under-ground water. During breeding, songsari in the culture tank were controlled to a light : dark photoperiod of 16 : 8 h and fed a mixed diet (Ewha oil & fat industry Co. Ltd., Korea) Water temperature of breeding tank ranged from 22 to 24°C. Eggs were collected from females, incubated in 10-L glass tank and checked for hatching.

Assay condition

In chronic exposure experiment, songsari were exposed to 50, 100 and 200 µg/l concentrations of BPA in a static-renewal system. Exposures took place in 1-L glass beaker filled with 900 ml of filtered Cheju Island under-ground water. The water quality parameter for the under-ground water were pH 8.1 and COD 0.8mg/l. The aqueous solutions of BPA were renewed every 72 h for the first month, and every 48 h thereafter, according to the methods described by Gray and Metcalfe (1997).

There were five exposure groups by two times of 40 fry each (total $n = 400$) at the start of the experiment. The five exposure groups were pure spring water and acetone / spring water in the control groups, and 50, 100, and 200 µg/l BPA in the treatment groups. Chronic exposure of songsari to BPA was initiated at 1 or 2 days after-hatching. Nominal BPA concentrations of 50, 100, and 200 µg/l were maintained by adding appropriate volumes (4.5, 9.0 and 18.0 µl) of BPA stock solution to the water in the aquaria. In the acetone (carrier) control, acetone alone (18.0µl) was added. The fish were maintained in a light : dark cycle of 16 : 8 h and were fed a mixed diet of 3-4 times daily for the duration of the experiment.

On the each 10, 20, 30, and 70 days after exposure, 8-30 individuals each were collected and the body weight and body length recorded. The examined total fish was 303 individuals. The length and weight of the fish were measured in 0.1 mm with the dissecting microscope, in 0.01g using an electron balance. The fish were then placed in tissue bottles and fixed in Bouin's fixative.

The fixed songsari were prepared for histological examination using histological procedures (dehydrated in ethyl-alcohol and embedded in paraffin). Fish were embedded whole in paraffin wax and sectioned (5 µm) with a microtome. The sections were stained using Hansen's haematoxylin and 0.5% eosin and examined under a light microscope. Sagittal-sections of the gonads were viewed with a microscope-monitor system. We calculated the proportion of sagittal sectional area of the gonad occupied by each germ cell type. The classification of the stages of oogenesis followed Yamamoto and Yoshioka (1964) and spermatogenesis followed Grier (1976).

Statistics

The analytical results were calculated by the method of χ^2 -test and two-way ANOVA.

Results and discussion

Sex differentiation and gonadal development

Just after hatching: 10 individuals were examined. Average body length of these individuals was 4.4 mm. The larvae still possessed the yolk. In sagittal-section, myotome, notochord and gut could be recognized. Between the myotome and the gut, germinal strand was located. The germ cells were visible in the anterior region of the primitive gonad. The germ cells were nearly ovoid shape and average diameter of these cells was 8 μm .

20 days after hatching: In female, the ovaries were composed of the oocytes of the chromatin-nucleolus stage and peri-nucleolus stage. Average diameter of the oocytes of the chromatin-nucleolus stage was 19 μm , and the oocytes of the peri-nucleolus was 25 μm . In male, the testes contained gonial cells alone. The gonial cells were nearly round or ovoid and average diameter of these cells was 7-8 μm .

30 days after hatching: The ovaries were composed of the oocytes of the chromatin stage and peri-nucleolus stage increased in numbers. In testis, a number of the spermatogonia and the spermatocytes were observed in the testicular lobule. In the process of the sex differentiation gonadal development was not different in the controls and BPA treatment groups until 30 days after hatching.

70 days after hatching: In the controls, the ovaries were composed of the oocytes of the chromatin-nucleolus, peri-nucleolus and yolk vesicle stage. The distribution(%) of its development stage were 13.1 \pm 2.1% and 86.9 \pm 2.1% in the control and 9.5 \pm 2.7%, 86.7 \pm 2.3% and 3.8 \pm 2.7% in the acetone (carrier) control respectively. Otherwise in the BPA treatment groups, the ovaries were composed of the oocytes of the chromatin-nucleolus, peri-nucleolus, yolk vesicle, yolk globule and mature stage. The frequency of oocyte development stage were 8.3 \pm 0.3%, 88.4 \pm 3.0% and 3.2 \pm 2.6% in BPA 50 $\mu\text{g/l}$, 4.3 \pm 0.1%, 89.6 \pm 2.8%, 3.9 \pm 1.2% and 2.2 \pm 1.6% in BPA 100 $\mu\text{g/l}$ and 3.9 \pm 0.2%,

80.1±1.5%, 8.4±3.1%, 5.5±2.5% and 2.1±1.9% in BPA 200µg/l respectively (Fig. 1).

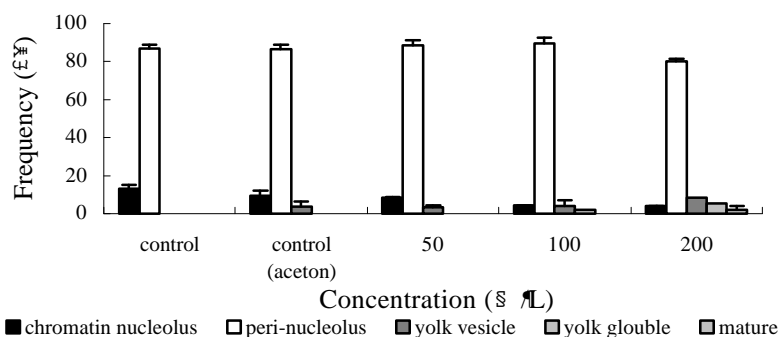


Fig. 1. Frequency of developmental stage of ovary in adult songsari, *Oryzias latipes* exposed to bisphenol A.

The developmental stage of oocyte came to be a yolk vesicle stage in the controls and came to grow yolks stage in the BPA treatment groups. Thus advanced development of oocytes in the ovary was observed from BPA treatment groups when compared to the controls. In sex differentiation of fishes, 17β-estradiol also advanced ovarian development in comparison with untreated females as observed in coho salmo (Foyle, 1993) and in pejerrey (Strüssmann et al., 1996).

In testis frequency of spermatogenesis stage is shown in Fig. 2. Frequency of spermatogonium, spermatocyte and spermatid in testis were 67.9±8.1%, 4.0±0.4% and 22.7±2.4% in the control and 73.5±3.7%, 9.1±5.2% and 17.4±1.5% in the acetone (carrier) control, 77.8±10.1%, 1.9±0.6% and 16.7±8.9% in BPA 50µg/l treatment group, 86.3±10.7%, 3.7±0.5% and 6.0±1.2% in BPA 100µg/l treatment group, 99.3±0.9%, 0.1±0.1% and 0.5±0.1% in BPA 200µg/l treatment group.

In these results, inhibition of development of spermatogenesis in the testis was observed from BPA treatment groups when compared to the controls. Nonylphenol and 17β-estradiol have severe effects on the testis and that the Sertoli cells might be affected in the eelpout (Christiansen et al., 1998). Oestrogen inhibits the differentiation of Leydig cells, Sertoli cells and early

formation of the spermatic duct in the European eel (Colombo and Grandi, 1995). In sexually developing fish, the pronounced effects on vitellogenin synthesis caused by exposure to the various estrogenic chemicals were accompanied by concomitant significant decreases in the testicular growth (Jobling et al., 1996)

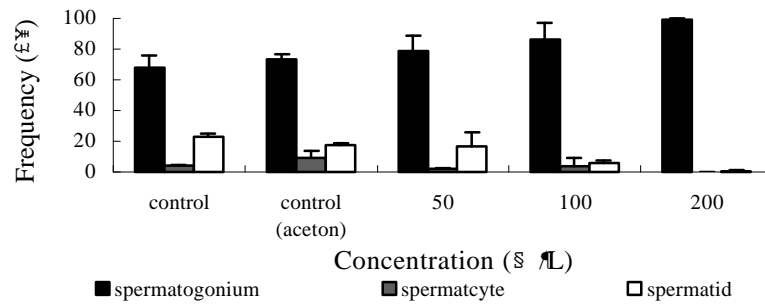


Fig. 2. Frequency of developmental stage of testis in adult songsari, *Oryzias latipes* exposed to bisphenol A

Sex ratio and growth

More females than males were identified in the BPA 50µg/l and 100µg/l treatments in comparison to the controls and BPA 200µg/l treatment (Table 1).

Table 1. Numbers of female and male songsari, *O. latipes* in the different groups from 10 to 70 days after hatching

Experimental group	Days after hatching				Total	Sex ratio (:)
	10 (:)	20 (:)	30 (:)	70 (:)		
Control	2:2	2:0	8:8	4:2	16:12	1:1
Control (acetone)	1:4	3:1	14:16	6:5	24:26	1:1
BPA 50 ppb	2:3	3:2	19:9	6:3	30:17	2:1
BPA 100 ppb	1:1	3:1	18:9	2:4	24:15	2:1
BPA 200 ppb	2:2	3:0	12:16	4:2	21:20	1:1

χ^2 test analysis indicated that sex ratios of female to male were 2 : 1 in the BPA 50 $\mu\text{g/l}$ and 100 $\mu\text{g/l}$ treatments ($P>0.05$). Fry of *O. latipes* exposed to 4.0 and 29.4 $\mu\text{g/l}$ 17 β -estradiol (Hartley et al., 1998) both exhibited 53% testis-ova or presumptive hermaphroditism, approximately 40% female and 5% male in each dose group. Gray and Metcalfe (1997) reported that fry of *O. latipes* exposed to 100 $\mu\text{g/l}$ of *p*-nonylphenol (NP) induced both the intersex state (i.e., testis-ova) in males as well as sex reversal (i.e., male to female), while exposure to a lower concentration of NP (50 $\mu\text{g/l}$) induced only testis-ova. But present histological analysis of 205 fishes uncovered an intersex individual.

The mean total lengths of songsari at sacrifice are presented in Table 2. Duncan's multiple test indicated that mean total lengths greater for fish in BPA 200 $\mu\text{g/l}$ treatment group in comparison another treatment groups. And BPA treated fish were slightly larger than untreated fish. Fish exposed to estrogenic compounds were larger than the control fish (Gray and Metcalfe, 1997; Moon, 1999).

Table 2. Changes of total length in the different groups at 10, 20, 30 and 70 days after hatching

Exp.	Days after hatching								
	10		20		30		70		
Group	Length (cm)	n	Length (cm)	n	Length (cm)	n	Length (cm)	Weight (g)	n
Control	0.65 \pm 0.04 ^a	10	0.83 \pm 0.11 ^a	10	1.13 \pm 0.14 ^b	30	1.69 \pm 0.20 ^b	0.04 \pm 0.02 ^b	8
Control (acetone)	0.66 \pm 0.04 ^a	10	0.85 \pm 0.04 ^a	10	1.15 \pm 0.14 ^b	30	1.74 \pm 0.22 ^b	0.06 \pm 0.03 ^{ab}	16
BPA 50 ppb	0.65 \pm 0.04 ^a	10	0.85 \pm 0.14 ^a	10	1.18 \pm 0.17 ^b	30	1.76 \pm 0.34 ^b	0.06 \pm 0.02 ^{ab}	16
BPA 100 ppb	0.65 \pm 0.05 ^a	10	0.88 \pm 0.12 ^a	10	1.19 \pm 0.12 ^b	30	1.87 \pm 0.29 ^b	0.06 \pm 0.02 ^{ab}	9
BPA 200 ppb	0.66 \pm 0.05 ^a	10	0.90 \pm 0.07 ^a	10	1.23 \pm 0.14 ^a	30	2.01 \pm 0.31 ^a	0.07 \pm 0.03 ^a	14

Values in the same column followed by a different letter are significantly different ($P<0.05$).

The results indicated that BPA exposed fish enhances ovary development and slows the development of testes. We do not know the mechanism underlying advancement of ovarian development and inhibition of testicular growth by

BPA. However based on these results, ovarian development and testicular growth may dependent on concentration of exposed to BPA.

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