GENOTOXICITY AND BIOCONCENTRATION OF POLYCYCLIC AROMATIC HYDROCARBONS AND HEAVY METALS IN *LEUCISCUS CEPHALUS* FROM PESCARA RIVER (ABRUZZO - ITALY): AN INTEGRATED APPROACH

ENQUÊTE ÉCOTOXICOLOGIQUE SUR LE FLEUVE PESCARA (ABRUZZES, ITALIE): GENOTOXICITE ET BIOCONCENTRATION DES HYDROCARBURES AROMATIQUES POLYCYCLIQUES ET DES MÉTAUX LOURDS DANS LE CHEVAINE

INDAGINE ECOTOSSICOLOGICA DEL FIUME PESCARA (ABRUZZO): GENOTOSSICITÀ E BIOCONCENTRAZIONE IN *LEUCISCUS CEPHALUS* DI IDROCARBURI POLICICLICI AROMATICI E METALLI PESANTI

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Abstract

The aim of the present study was to investigate the water quality and the ecosystem health of a segment of Pescara river (Abruzzo-Italy) with the aid of an integrated approach. Sixteen priority polycyclic aromatic hydrocarbons and heavy metals were determinated in water and chub samples in order to calculate the bioconcentration factor. Biochemical, physical and microbiological characterisation were also carried out on water samples. Moreover, real and synergic effects of water pollution on biota were investigated by genotoxicological tests. Water samples were tested by *Salmonella* mutagenicity assay, whereas micronucleus assay on chub was used to evaluate environmental genotoxic effects on fish. The characterisation of water samples did not reveal any relevant contamination of Pescara river, but some analytical results obtained for biota were remarkable. In particular, the comparison between the site downstream from the industrial area of Chieti and the control site showed significant differences in chub micronuclei frequencies.

Keywords: *freshwater; bioconcentration; polycyclic aromatic hydrocarbons; heavy metals; Ames test; micronucleus test*

Résumé

L'objectif de cette recherche était d'étudier certains aspects de la qualité de l'eau et de la santé des écosystèmes d'une partie du fleuve Pescara (Abruzzes, Italie). Nous

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avons d'abord analysé les paramètres physico-chimiques et microbiologiques de l'eau, puis nous avons déterminé la bioconcentration des hydrocarbures aromatiques polycycliques et des métaux lourds dans le chevaine (*Leuciscus cephalus*). En outre, nous avons réalisé des tests de génotoxicité de l'eau pour évaluer les effets synergiques sur les organismes vivants. En particulier, nous avons évalué la mutagénicité sur *Salmonella* et utilisé le test du micronoyau sur *L. cephalus* comme biomarqueur. Les résultats ont montré que la qualité de l'eau du fleuve Pescara n'est pas encore compromise; mais certains résultats des analyses biologiques indiquent une altération dans les zones d'étude. En particulier, l'échantillon de chevaines prélevé en aval de la zone industrielle de Chieti présente une augmentation de la fréquence des micronoyaux par rapport aux témoins.
Mots-clés: *eaux de surface; bioconcentration; hydrocarbures aromatiques polycycliques; métaux lourds; test d'Ames; test du micronoyau*

Riassunto

Obiettivo del lavoro è stato indagare alcuni aspetti della qualità delle acque e della salute ecosistemica di un segmento del fiume Pescara (Abruzzo). E'stata dapprima effettuata una caratterizzazione dei siti mediante l'analisi di parametri chimico-fisici e microbiologici delle acque, quindi è stata determinata la bioconcentrazione nel cavedano di idrocarburi policiclici aromatici e metalli pesanti. L'analisi chimica è stata accompagnata da test genotossicologici per valutare gli effetti sinergici sul biota. In particolare, è stato eseguito il test del micronucleo su *L. cephalus* come biomarker e il test di mutagenesi su *Salmonella*. Dallo studio è emerso che la qualità delle acque del fiume Pescara non è ancora compromessa; indicazioni su un'alterazione dei siti di studio risultano invece dall'analisi delle componenti biologiche. In particolare, il campione di cavedani raccolto a valle della zona industriale di Chieti presentava una maggiore incidenza di cellule micronucleate rispetto a quello raccolto nel sito controllo.
Parole chiave: *acque superficiali; bioconcentrazione; idrocarburi policiclici aromatici; metalli pesanti; test di Ames; test del micronucleo*

Introduction

Pescara river is the longest river in Abruzzo, central-eastern Italy; it has been a crossroad of peoples, history and commerce. The river gets across a highly anthropized area of the region with several urban settlements and industrial facilities. In 2008, it was also included in the contaminated Site of "Bussi sul Tirino", where in 2007 was discovered the biggest illegal dump of toxic waste in Europe just in the left river bank. For this reason, a comprehensive study based on the ecotoxicological assessment of a portion of Pescara river from the viewpoint of the ecosystem health, is here reported. Chemical analyses and genotoxicological tests on water and fish samples are included.

Materials and methods

Study area and collection. Surface water and fishes were sampled in July and December 2008, when maximum and minimum stream flow values respectively are observed, at three different sites along Pescara river. Nora river, a tributary of Pescara river, was chosen as control site, as shown in Fig.1.

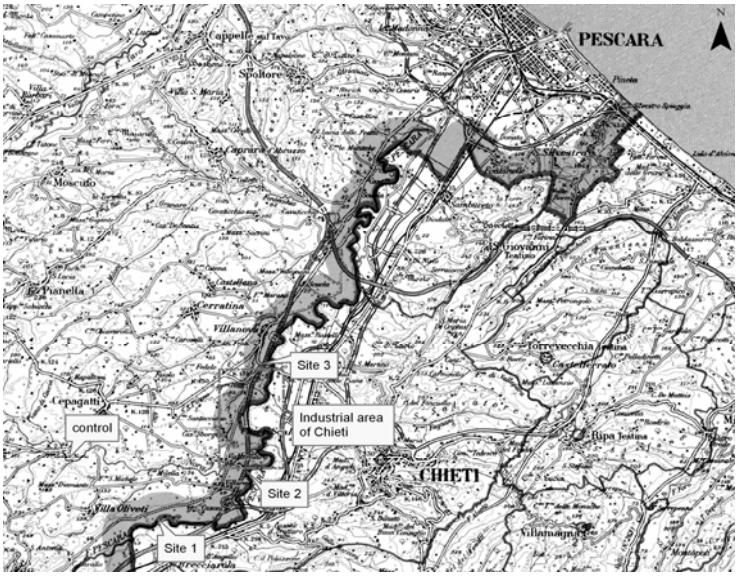

Figure 1

Samplig sites in the study area of Pescara river.

Fish samples were caught by electrofisher and kept alive for 24 hours for the micronucleus (MN) test. Chub was used as bioaccumulator, according to its bio-ecological characteristics. Moreover, in order to evaluate the global quality of the study sites, the extended biotic index (EBI) was calculated, as modified by Ghetti (APAT/IRSA method n. 9010,2003), using the number of species, the abundance of individuals, the diversity of macroinvertebrates community. Chemico–physical parameters of surface waters (pH, temperature, conductivity, total dissolved solids, dissolved oxygen) were determinated *in situ* using different multiprobes.

Chemical and microbiological analyses. For each site, representative samples of fish tissue were prepared for the 16 priority polycyclic aromatic hydrocarbons (PAHs) determination, according to EPA Method 3540C (Anyakora et al.,2005; Jones et al., 2005; Vives et al., 2004). Samples (10 g) were blended with anhydrous sodium sulfate until a dry homogenate was obtained, and then Soxhlet extracted using an acetone- dichloromethane mixture (1:1, v/v). The extracts were dried through a column containing anhydrous sodium sulfate before their purification by a standard column silica gel cleanup (EPA Method 3630C). Surface water samples were prepared for PAHs analysis by solid phase extraction on Oasis® HLB (Waters, Milford, MA, USA) cartridges. PAHs in water and fish extracts were determined by gas chromatography-mass spectrometry (GC-MS).

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Heavy metals (Hg, Pb, Cd, Cr, As) determination in water and fish samples (after digestion) was carried out by atomic absorption spectroscopy (AAS), according to APAT-IRSA-CNR methods n. 3080, 3120, 3150, 3200, 3230 (2003), ISTISAN method n. 34/96 and UNI EN method 14546/05.
Moreover, in order to better evaluate the contamination of the study sites, water samples were analysed to determine ionic species, aromatic and chlorinated solvents, oxygen demand (BOD_5 and COD) and fecal bacteria community (APAT/IRSA methods n. 7010 and 7040,2003). In particular, ionic species were analysed by ionic chromatography (APAT/IRSA methods n. 3030 and 4020,2003) and solvents were determined by static headspace GC-MS.

Ecotoxicological tests. Water samples mutagenicity was evaluated by *Salmonella typhymurium* test (Ames test). The plate incorporation assay was performed with and without metabolic activation (S9 mix), using TA98 and TA100 as tester strains. A negative (dimethylsulfoxide) and two positive (sodium azide and 2-aminofluorene) control samples were included in each essay. Prior to the test, water samples were preconcentrated on Amberlite XAD-4 resin (Supelco, Bellefonte, PA, USA). A blank sample (BLK, distilled water) was also extracted. MN test was carried out on Chub erythrocytes and used as biomarker of genotoxicological environmental contamination (Al-Sabti et al., 1995). Peripheral blood samples were obtained from the caudal vein of the specimens and smeared onto precleaned slides. After fixation in pure methanol for 10 min, the slides were stained with 10% Giemsa. Three slides for each fish were prepared, and from each slide 2000 polychromatic erythrocytes were scored under 100X magnification. Small, no refractive, circular, or ovoid chromatin bodies, displaying the same staining and focusing pattern as the main nucleus, were scored as micronuclei according to the APAT/IRSA method (2005). Genetic damage was determined as MN frequency.

Results

The results obtained from chemical and biological analyses of water and fish samples collected in July 2008 are summarised in Tables 1 and 2. Analyses of samples collected in December 2008 gave results in agreement with those of July. The bioconcentration factor (BCF) of each chemical substance, calculated as the ratio of the concentration in the fish tissue (ppm) to its concentration in water (ppm), is also reported.
The organic solvents in surface water were under their limit of detection (0.5 µg/l). As expected, heavy metals and PAHs levels were higher in Chub tissues than in water, although they were low and within the limits of the Italian regulations.
The results of the genotoxicological tests obtained in this study are presented in Figures 2 and 3. The mutagenicity ratio, calculated as the ratio of revertant colonies in the sample to those in the negative control, was below 2, indicating the lack of mutagenicity of water samples (Kutlu et al., 2004; Siddiqui et al.,2003; Pereira et al., 2007), while, the MN test showed a genotoxic effect on fish. In fact, a more careful comparison between the site 3, downstream from the industrial area of

Chieti, and the control site revealed significant differences in MN frequencies (p<0.05), evaluated by nonparametric statistic analyses (Dunn method).

Table 1 - *Surface water characterisation of Pescara River (July 2008).*

PARAMETER	**Control site**	**SITE 1**	**SITE 2**	**SITE 3**
T (°C)	16.5	18.2	16.7	18.8
pH	8.3	8.2	7.9	8.2
% O_2	105	119	102	118
Conductivity (µS/cm)	581	400	446	455
TDS (ppm)	291	204	222	227
EBI Value	II	III	-	III
EBI Judgement	Moderately altered environment	Altered environment	-	Altered environment
BOD_5 (ppm)	0.9	1.6	1.4	1.7
COD (ppm)	< 10	15	14	10
MICROBIOLOGICAL ANALYSES (UCF/100 ml)				
E. coli	411±28	4400±93	13500±163	5500±104
Coliforms	1533±55	9000±133	12500±156	16000±177
Enterococcus	433±29	550±31	3100±78	1150±47
IONIC SPECIES (mg/l)				
F^-	0.27	0.10	0.12	0.15
Cl^-	27.86	7.96	9.00	13.60
Br^-	< 0.50	< 0.50	< 0.50	< 0.50
NO_2^-	< 0.50	< 0.50	< 0.50	< 0.50
NO_3^-	< 0.50	2.31	2.80	3.97
PO_4^{3-}	< 0.50	18.97	< 0.50	< 0.50
SO_4^{2-}	93.98	< 1.00	23.15	37.70
Li^+	0.02	0.01	< 0.01	< 0.01
Na^+	37.02	9.26	9.84	14.44
NH_4^+	< 0.10	< 0.10	< 0.10	< 0.10
Mg^{2+}	28.60	22.32	22.39	25.69
K^+	5.50	1.98	2.04	2.50
Ca^{2+}	89.70	78.13	80.78	84.88
Sr^{2+}	0.61	< 0.50	< 0.50	< 0.50
Ba^{2+}	< 1.00	< 1.00	< 1.00	< 1.00

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Table 2 - *PAHs and heavy metals in water and fish samples from Pescara River (July 2008).*

	Control site			Site 1			Site 2			Site 3		
Metals	Water (mg/l)	Fish (mg/kg)	BCF	Water (mg/l)	Fish (mg/kg)	BCF	Water (mg/l)	Fish (mg/kg)	BCF	Water (mg/l)	Fish (mg/kg)	BCF
Hg	< 0.005	<0.05	-	< 0.005	0.08	-	< 0.005	0.06	-	< 0.005	<0.05	-
Pb	<0.010	<0.05	-	<0.010	<0.05	-	<0.010	<0.05	-	<0.010	<0.05	-
Cd	< 0.005	<0.010	-	< 0.005	<0.010	-	< 0.005	<0.010	-	< 0.005	<0.010	-
Cr	<0.010	0.082	-	<0.010	0.049	-	<0.010	0.055	-	<0.010	0.061	-
As	<0.010	0.15	-	<0.010	0.13	-	<0.010	0.12	-	<0.010	0.44	-
PAHs	Water (mg/l)	Fish (mg/kg)	BCF	Water (mg/l)	Fish (mg/kg)	BCF	Water (mg/l)	Fish (mg/kg)	BCF	Water (mg/l)	Fish (mg/kg)	BCF
Naphthalene	0.028	<0.05	-	0.006	<0.05	-	0.025	<0.05	-	0.007	<0.05	-
Acenaphthylene	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-
Acenaphthene	<0.001	<0.05	-	0.013	0.30	23	0.012	<0.05	-	0.011	<0.05	-
Fluorene	0.005	7.09	1418	0.004	6.83	1707	0.003	7.31	2437	0.005	7.77	1554
Anthracene	0.020	7.23	361	0.013	7.81	601	0.015	7.10	473	0.010	7.57	757
Phenantrene	0.005	2.06	412	0.021	2.23	106	0.003	2.44	813	0.002	2.19	1095
Fluoranthene	0.001	0.91	0.91	0.002	1.08	540	0.002	0.75	375	0.001	0.82	820
Pyrene	0.03	0.93	310	0.004	1.96	490	0.003	0.87	290	0.002	0.69	345
Benzo[a]anthracene	<0.0005	<0.0005	-	<0.0005	<0.025	-	<0.0005	<0.025	-	<0.0005	<0.025	-
Chrysene	<0.0005	0.24	-	<0.0005	<0.025	-	<0.0005	<0.025	-	<0.0005	<0.025	-
Benzo[b]fluoranthene + Benzo[k]fluoranthene	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-
Benzo[a]pyrene	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-
Indeno[1,2,3-cd]perylene	0.001	<0.05	-	0.001	<0.05	-	0.001	<0.05	-	0.001	<0.05	-
Dibenzo[a,h]anthracene	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-
Benzo[g,h,i]perylene	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-	<0.001	<0.05	-
Σ(PAHs)	0.062			0.062			0.063			0.039		

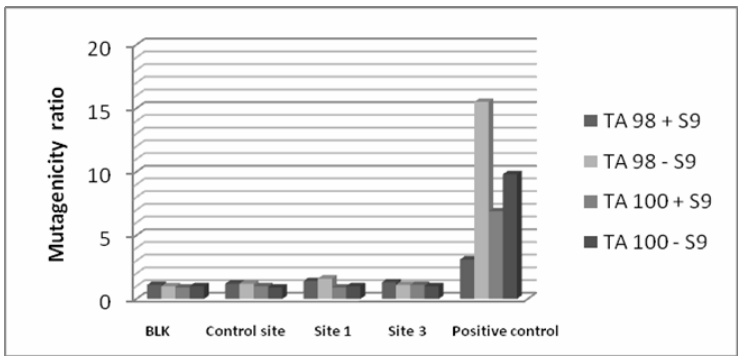

Figure 2

Mutagenicity analysis of concentrated water samples from Pescara River.

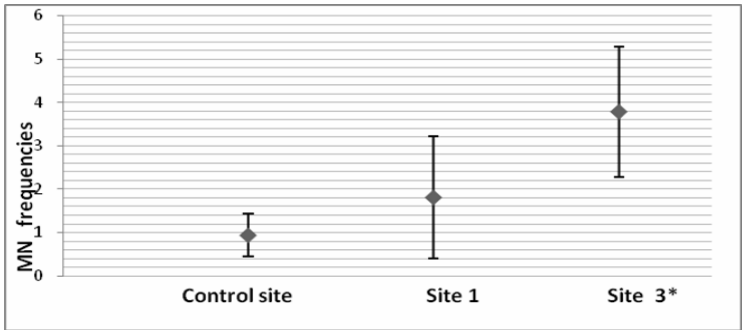

Figure 3

*Spontaneous frequency of micronucleated erythrocytes/2000 cells from Pescara River (*p <0.05 respect to control).*

Discussion and conclusions

A chemical and ecotoxicological study of Pescara river was carried out for the first time and is here reported. The characterisation of water samples did not reveal a relevant contamination of the river. Whereas, biological and ecotoxicological tests showed the negative impact of environmental pollutants on the biological community. Among these, *EBI* showed an "Altered environment", microbiological analyses indicated a fecal contamination of water and the MN test revealed genotoxic effects on fish, claiming the need for actions aimed at the monitoring and reduction of pollution of Pescara river. Anyway, the results obtained for biota need to be deepened in further investigations, also extending the study to other sections of the river.

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