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Genetic Toxicology and Environmental Mutagenesis

Mutagenicity (micronucleus test in *Vicia faba* root tips), polycyclic aromatic hydrocarbons and heavy metal content of sediments collected in Tiber river and its tributaries within the urban area of Rome

Sandra Minissi [a,*], Daniela Caccese [a], Fabio Passafiume [b], Angiolina Grella [b], Eleonora Ciccotti [a], Marco Rizzoni [a]

[a] *Dipartimento di Biologia, Università di Roma 'Tor Vergata', Viale della Ricerca Scientifica snc, 00133 Rome, Italy*
[b] *Dipartimento di Sanità Pubblica, Università di Roma 'Tor Vergata', Via di Tor Vergata, 00133 Rome, Italy*

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Abstract

Sediments collected in Tiber river and in its main tributary water courses within the urban area of Rome were tested for mutagenicity by means of *Vicia faba* root tips micronucleus (MN) test. Representative samples were scored for micronucleus generating events (chromosome/chromatid loss and fragments) too. Sediments were assayed for content of the thirteen most important chemicals of polycyclic aromatic hydrocarbon (PAH) group and for some heavy metal ions (Cd, Cr, Cu, Ni, Pb, Zn). Samples were collected in four tributary rivers (Prima Porta, Acqua Traversa, Aniene and Magliana) just before their confluence with Tiber river and at different stations along the Tiber river itself upstream and downstream the sites of confluence of the sampled tributaries. All samples were collected in July 1992. An alarming level of mutagenicity was reached in most of the tested stations, with an effect comparable to an X-rays exposure up to 0.4 Gy. Chemical analysis showed that the total amount of identified PAHs ranged from 4.5 to 625.2 ng/g of dry matrix in the different stations and the total amount of heavy metals ranged from 130 to 570 ppm. Tiber mutagenicity is likely to be mainly due to local factors such as the confluence of a small polluted tributary rather than to large scale effect due to an upstream–downstream relationship.

Keywords: Micronucleus; *Vicia faba*; PAH; Heavy metal; Natural matrix

1. Introduction

Municipal waste waters, industrial and agriculture effluents, but also natural agents [1], can account for mutagenicity of environmental matrices (air, water and soil). In recent years, the contamination of natural environments is becoming an issue of increasing concern leading to the development of short-term bioassays with the aim of testing environmental complex mixtures.

In order to test complex mixtures, three main approaches may be followed: (1) laboratory ap-

* Corresponding author. Tel.: +39-06-72594812; Fax: +39-06-2023500; E-mail: minissi@bio.uniroma2.it

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proach; (2) in situ approach; (3) intermediate approach. By the first approach, a single or few chemicals are extracted (and in some cases concentrated) and tested under laboratory conditions by means of standardised bioassays [2,3]. By the second approach, complex mixtures are tested directly 'on field' through the analysis of damage induced in naturally exposed organisms (bioindicators) [4–6]. By the 'intermediate' approach, the crude sample is tested, without any previous treatment, under laboratory conditions by means of a well-known and standardised bioassay. Such an approach has been successfully applied using both plants [7–9] and aquatic animals [10–12]. Furthermore, the 'intermediate' approach has the advantage that most of the variables are under control and that the 'real' mutagenicity of the complex mixture can be studied.

Since 1989, we have been studying Tiber river mutagenic pollution within the urban area of Rome (Italy). In our studies, two experimental approaches have been successfully followed scoring micronucleus (MN) frequency in different biological systems: *Vicia faba* root tips exposed to water or sediment under laboratory conditions, according to the 'intermediate' approach [9,13]; peripheral erythrocytes of fish captured in natural environments following the in situ approach [6].

The present paper shows results from a campaign (July 1992) in which Tiber river's tributary water courses were tested for mutagenicity by means of *V. faba* MN test. The first investigated tributary, Prima Porta, is at the entry of the urban area; the second, Acqua Traversa, is close to a small airport inside the city of Rome; the third, Aniene is the biggest tributary among the tested ones; the last one, Magliana, is immediately outside Rome, 20 km from the sea.

Micronucleus test was performed on *V. faba* root tips exposed to sediments collected in the tributaries just before their confluence with Tiber river. In order to study their effects on Tiber river, sediments were also collected at different stations along Tiber river itself corresponding to the site immediately upstream and downstream (when possible) the sites of confluence of the sampled tributaries. Some representative samples were also scored for micronucleus generating events at ana-telophase: fragments and lagging chromosomes/chromatids. All samples were collected in July 1992. Sediments were preferred to water samples because it is known that some genotoxic metals and hydrophobic chemicals are preferentially sequestrated in sediments themselves. Furthermore, sediments have a more stable composition than flowing water because they accumulate and gradually released chemicals in the environment.

The same samples were analysed for the content of some potentially mutagenic heavy metal ions (Cd, Cr, Cu, Ni, Pb, Zn) and some selected polycyclic aromatic hydrocarbons (PAHs). PAHs and heavy metals are ubiquitous contaminants of natural environments and most of them are potent mutagens and carcinogens [14,15]. Moreover, PAHs are lipophilic chemicals which are readily taken up by aquatic organisms and subject to bioaccumulation through the food chain.

2. Materials and methods

2.1. Sampling

Samples were collected in July 1992 in four Tiber river's tributaries within the urban area of Rome, immediately before their confluence with the main river: (1) Prima Porta (P.P.); (2) Acqua Traversa (A.T.); (3) Aniene (A.), and (4) Magliana (M.). On Tiber river, samples were collected at different stations corresponding to the site immediately upstream and downstream (when possible) the sites of confluence of the sampled tributaries. Sampling stations were: (1) upstream Prima Porta (u.P.P.); (2) Castel Giubileo, downstream Prima Porta (d.P.P.); (3) upstream Acqua Traversa (u.A.T.); (4) 'Urbe' airport, downstream Acqua Traversa and upstream Aniene (d.A.T./u.A.); (5) Ponte Tor di Quinto, downstream Aniene (d.A.) and (6) downstream Magliana, (d.M.) (Fig. 1).

At each site, 500 g of sediment were collected at a distance of about 1 m from the edge of the river; 400 g were immediately used for mutagenicity testing whilst 100 g were kept at −20°C until chemical analysis was performed.

2.2. Cytogenetic analysis

Fifty *V. faba* seeds per point were placed for germination on a substrate made with sediment sam-

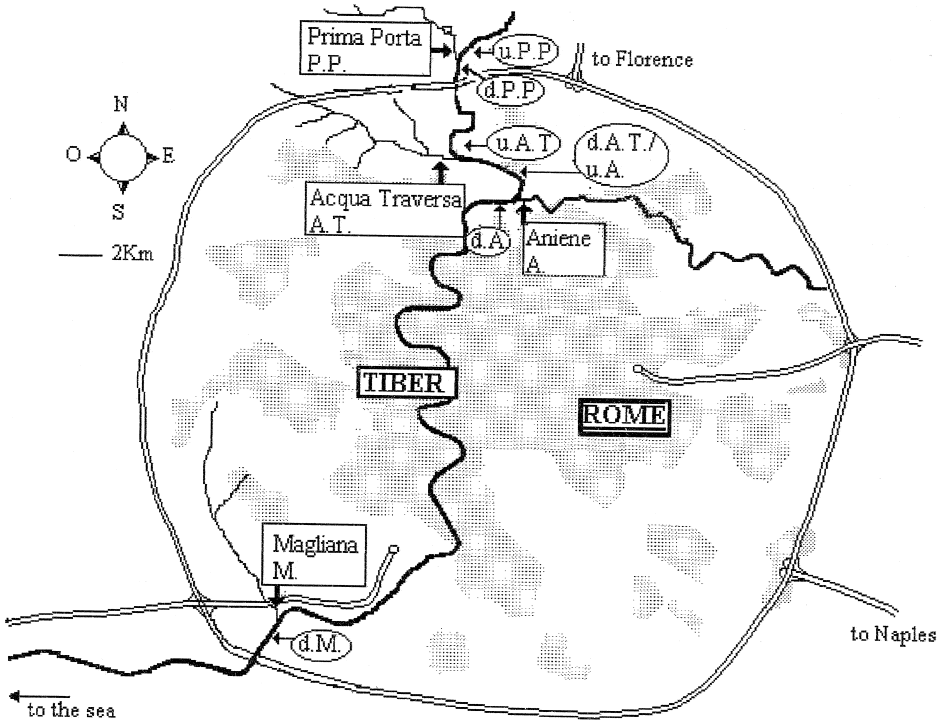

Fig. 1. A map of Rome. The sampling places in tributary water courses are represented by the large arrows (Prima Porta: P.P.; Acqua Traversa: A.T.; Aniene: A.; Magliana: M.); the ones in the Tiber river, upstream and downstream the confluence of tributaries, are represented by the small arrows (u.P.P., d.P.P.; u.A.T., d.A.T./u.A., d.A.; d.M.). Grey areas represent densely urbanised areas; black-edged empty lines represent main roads; black full lines represent the Tiber river and the tributaries studied in this paper.

ple and synthetic clay (vermiculite) mixed in a ratio of approximately 1:1. As a negative control, synthetic clay was mixed up with tap water. Germination was carried out at 20°C in the dark with 100% moisture for 4–5 days. Primary roots were fixed in methanol/acetic acid (3:1 ratio), Feulgen stained, squashed on slides in 45% acetic acid and permanently mounted in Eukitt. The whole tip (meristematic and F1 regions) was preferred to only F1 as suggested by Ma et al. [16] to avoid to introduce an error in micronucleus estimation due to mistakes in choosing the region to be analysed. Scoring for counting a MN was based on generally accepted criteria for MN identification [17]. Micronuclei were considered only when their diameter did not exceed 2/3 of the main nucleus [18], showed non-refractility and they were localised within the cell wall and in the cytoplasmic area surrounding the main nucleus. Cells with their MN in contact with the main nucleus or with more than two MN were excluded, as they could be the result of nuclear extrusions or degenerative processes. However, the observed frequency of cells with two MN was very low accordingly with a Poisson's distribution.

In order to avoid underestimation of micronucleus frequency due to impaired cell proliferation rate, cytogenetic analysis was performed only in root tips with a mitotic index ranging from 10 to 25%. Ten tips for each experimental group were scored blind. Micronucleus frequency was assessed in 5000 cells/tip; in the same tips ana-telophases/mitoses ratio was estimated in 5000 cells to verify whether there were heavy spindle damages which could interfere with micronucleus origin. Samples which exhibited a low micronucleus frequency (10–11 MN/5000 cells: upstream Acqua Traversa (u.A.T.) and upstream Prima Porta (u.P.P.)), a medium one (34–45 MN/5000 cells: Acqua Traversa (A.T.) and downstream Prima Porta (d.P.P.)) and a high one (85–86 MN/5000 cells: downstream Acqua Traversa/up-

stream Aniene (d.A.T./u.A.) and downstream Magliana (d.M)) were scored for micronucleus generating events analysing, in each sample, 100 anatelophases for chromosome/chromatid loss and fragments.

Student's *t*-test was used to determine significant differences between each experimental point and control at 0.01 level of significance. A linear regression analysis was carried out between the frequencies of generating events and those of micronuclei.

2.3. Chemical analysis

For heavy metals (Cd, Cr, Cu, Ni, Pb, Zn) quantification dried sediment samples were mineralised in the presence of a 4:1 chloride acid–nitric acid mixture for 12 h. Residues were dried, filtered, suspended in deionized water and analysed by atomic absorption spectrometry using a Perkin-Elmer 5000 instrument equipped with a HGA 500 graphite furnace.

Aliquots for PAH analysis were obtained from 24 h benzol-soxhlet extracts of dried sediment samples. Benzenic extracts were dried and eluted through an alumina packed column with 10 ml of cyclohexane. Eluates were dried and made up to 600 μl volume with acetonitrile. Such samples were analysed for PAHs content by means of HPLC technique using both ultraviolet (fixed wavelength at 225 nm) and fluorescence (excitation at 280 nm, emission at 390 nm cut off filter) detection. Peak identification of PAHs was performed using retention time matching technique and spectral analysis of each detected peak. Quantification of PAHs was achieved by comparing our results with an external standard curve obtained by scalar dilution of standard solutions of the 13 most important chemicals of the PAH group (SUPELCO EPA 610 PAH mixture): Fluorene, Phenanthrene, Anthracene (A), Fluoranthene (Fl), Pyrene (P), Benzo(a)anthracene (B(a)A), Chrysene (Chr), Benzo(b)fluoranthene (B(b)Fl), Benzo(k)fluoranthene (B(k)Fl), Benzo(a)pyrene (B(a)P), Dibenz (a,h)anthracene, Benzo(g,h,i)perylene and Indeno(1,2,3cd)pyrene (IP).

2.4. Comparison between mutagenicity and chemical pollution

A multiple linear regression analysis was performed assuming pollutant concentrations as independent variables and micronucleus frequencies as dependent ones. Furthermore, a Factorial Correspondence Analysis (FCA) [19] was performed, using both chemical concentration data and micronucleus frequencies as descriptors and sampling sites as observations, to verify whether sampling stations may be grouped on the basis of the relationship between pollutant concentration and induced micronucleus frequency. For both the analyses, the concentrations of PAHs which have a well-known carcinogenic activity were used [20], their values pooled together and expressed as toxicological Benzo(a)pyrene Equivalent (BPE) units. A BPE unit is the amount of a specific PAH which has the same carcinogenic potency of a weight unit of B(a)P. Actually, according to Zapponi and Valente [21], the total carcinogenic potency of Benzo(a)anthracene, Benzo(b)fluorantene, Benzo(k)fluorantene, Benzo(a)pyrene and Indeno(1,2,3cd)pyrene may be expressed as a sum of B(a)P toxicity equivalents (BPE units), which are calculated multiplying the weights of the different PAHs by appropriate conversion factors (BPEFs). The latter are B(a)A, BPEF = 0.006; B(b + k)Fl, for each isomer, BPEF = 0.076; B(a)P, BPEF = 1 and IP, BPEF = 0.08.

3. Results

3.1. Mutagenicity test

The results of *V. faba* micronucleus test are summarised in Table 1. All sampled tributary rivers show a significant and remarkable ($P < 0.01$) higher micronucleus frequency than control. Moreover, with the remarkable exception of Aniene river, tributaries seem to increase Tiber river mutagenicity at the site downstream their confluence as it can be pointed out by comparing the micronucleus frequency at the upstream site with that recorded at the site downstream the confluence.

If present data are compared to other data of 1992, published elsewhere [22] and cited by Minissi and Lombi [13], a noticeable variation can be pointed out even if within the same range of values. This variation can be explained taking into account that

Table 1
Average micronucleus frequency/5000 cells and ana-telophases/mitoses ratio in root tips of *Vicia faba* exposed to sediments collected in July 1992 in Tiber and in its main tributary water courses within the urban area of Rome

Sampling station[a]	% Ana-telophase/mitoses ratio ± SE	Micronucleus frequency/5000 cells ± SE
Control	6.2 ± 0.4	7.3 ± 1.5
u.P.P.	7.3 ± 0.9	11.5 ± 3.5
P.P.	6.1 ± 0.4	13.8 ± 1.5[b]
d.P.P.	6.8 ± 0.6	45.5 ± 12.7[b]
u.A.T.	6.1 ± 0.5	10.1 ± 1.6
A.T.	5.9 ± 0.4	34 ± 4.1[b]
d.A.T./u.A.	5.6 ± 0.4	85.3 ± 16.7[b]
A.	5.9 ± 0.6	89 ± 12.0[b]
d.A.	9.8 ± 1.3	22.5 ± 5.1[b]
M.	6.8 ± 0.7	25.8 ± 5.2[b]
d.M.	4.0 ± 1.1	86.6 ± 12.6[b]

Ten root tips per experimental group, 5000 cells for each tip were scored.
[a] For station acronyms, see text.
[b] The observed MN frequency is significantly higher ($P < 0.01$) than the control value, using Student's *t*-test.

sediment samples described in the present paper were collected 1 month later than the other ones suggesting a rapid change in mutagenic pollution.

3.2. Micronuclei and generating events

In all tested samples, the ratio ana-telophases/mitosis is rather constant (Table 1). Therefore, a damage to the spindle so as to slow or block mitotic progression (cytotoxicity) with the consequent formation of micronucleated cells has not been induced. In such conditions, it becomes possible to make a direct comparison between the frequency of chromosome aberrations at ana-telophase and micronucleus frequency. It is well known that acentric fragments and lagging chromosomes are directly involved in MN origin. In order to investigate this relationship, linear regression analysis was carried out assuming as independent variable the frequency of fragments and laggards and the frequency of micronuclei as dependent one (Fig. 2). Because of the high value of the correlation coefficient ($r = 0.9$), a strong correlation between the two events was demonstrated.

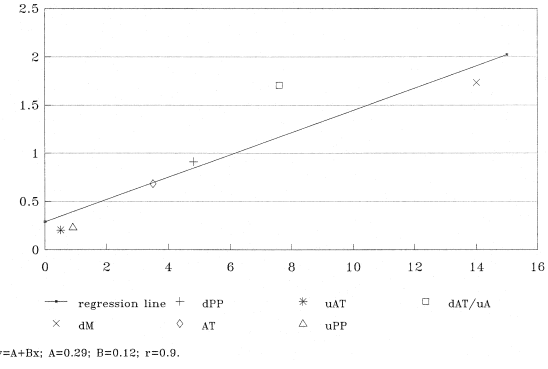

Fig. 2. Relationship between the frequencies of micronuclei in interphase and those of their generating events in ana-telophase (acentric fragments + lagging chromosomes). The straight line is the linear regression line; the marked points indicate the sampling stations (see text).

3.3. Chemical analysis

Results of chemical analysis for heavy metals content are summarised in Table 2. Concentrations of Cu and Cd are comparable with the levels measured in nonindustrial areas by different authors [23,24]; as to the capability to induce a significant increase in micronucleus frequency in *V. faba* root tips, Cd concentrations are much lower and Cr concentrations are higher than those required to show a mutagenic effect [25].

Table 3 summarises sediment content of some of the most representative compounds of the PAH

Table 2
Chemical analysis for heavy metal content of sediments collected in July 1992 in Tiber and in its main tributary water courses within the urban area of Rome

Sampling station [a]	Metal concentration (ppm)					
	Cd	Cr	Cu	Ni	Pb	Zn
u.P.P.	0.1	47.3	27.2	9.5	25.5	89.4
P.P.	0.5	54.2	45.5	32.5	19.5	417.6
d.P.P.	0.4	34.5	26.9	33.5	17.3	92.4
u.A.T.	0.1	35.8	19.4	19.8	29.1	88.1
A.T.	0.3	30.5	13.4	11.7	21.6	53.4
d.A.T./u.A.	0.1	38.1	20.1	27.3	20.1	114.7
A.	0.6	38.9	43.1	13.0	43.1	156.9
d.A.	0.1	26.3	16.0	15.5	14.10	78.0
M.	0.1	18.2	13.3	3.6	12.4	222.1
d.M.	0.1	36.4	20.6	23.9	17.7	114.1

[a] For station acronyms, see text.

Table 3
Chemical analysis for polycyclic aromatic hydrocarbon (PAH) content of sediments collected in July 1992 in Tiber and in its main tributary water courses within the urban area of Rome

Station[a]	PAH content (ng/g dry matrix)[b]												
	F	Ph	A	Fl	P	B(a)A	Chr	B(b)Fl	B(k)Fl	B(a)P	DB(a,h)A	B(g,h,i)Per	IP
u.P.P	3.6	31.2	6.2	86.3	46.8	n.d.	64.5	86.3	28.3	52	n.d.	66.5	54.1
P.P.	n.d.	23.1	2.2	46.6	23.5	12.7	17.5	n.d.	8.1	n.d.	20	n.d.	11.3
d.P.P.	n.d.	45	15.1	85.5	71.2	38.8	57	75	31.2	34.3	97.8	16.8	57.5
u.A.T.	n.d.	1.9	n.d.	n.d.	n.d.	n.d.	2	n.d.	0.6	n.d.	n.d.	n.d.	n.d.
A.T.	n.d.	4.6	n.d.	9.5	5.8	4	4	7.4	2.3	4.4	8.3	n.d.	n.d.
d.A.T./u.A.	n.d.	8.7	n.d.	20	14.2	11	13	23.5	9.2	15.8	54.7	23.1	17.1
A.	n.d.	5.5	1.3	10.2	8.3	5.4	6.2	6.5	2.7	6	11.6	n.d.	n.d.
d.A.	n.d.	13.7	n.d.	14.8	8	4.8	6.5	9.4	3.5	n.d.	11.1	n.d.	n.d.
M.	n.d.	31.4	5.7	70	53	29.5	34.5	39.5	14.7	29	46.2	10	20.1
d.M.	2.3	2.6	n.d.	4.4	n.d.	1.9	2.3	3.8	0.6	n.d.	n.d.	n.d.	n.d.

[a] For station acronyms, see text.
[b] For PAH acronyms, see text.

group. Measured PAH concentrations in the present paper lie within the range of background PAH levels in soil (100–1000 ng/g dry weight) [26].

4. Discussion

4.1. Comparisons with preexisting data

As mentioned before, we have been studying Tiber river mutagenicity since 1989, using the same test system, with a first campaign in 1989–1990 [9], a second campaign in 1992 (present paper and Ref. [22]) and a third campaign in 1995 [13]. Results obtained in different campaigns can be directly compared at three sampling stations along Tiber river: Castel Giubileo, Ponte Tor di Quinto and Ponte della Magliana. Each of these stations shows a similar behaviour during the years. Actually, in the first campaign, no significant MN induction was observed with sediment samples collected both at Castel Giubileo and Ponte Tor di Quinto (micronucleus frequencies per 5000 cells were 10 and 10.4, respectively) [9]. Also in the third campaign no significant induction of MN was observed at Castel Giubileo, at Ponte Tor di Quinto and at Ponte della Magliana (micronucleus frequencies per 5000 cells were 9.8, 9.3 and 11.2, respectively) [13]. Present results from the second campaign, show a remarkable worsening of mutagenic pollution of Tiber river compared to both the campaigns at Castel Giubileo and at Ponte Tor di Quinto, and compared to the third campaign at Ponte della Magliana (micronucleus frequencies per 5000 cells are 45.5, 22.5 and 86.6, respectively). Such a mutagenic effect is comparable to an X-ray exposure ranging from 0.1 to 0.4 Gy [27]. These results suggest a worsening between 1990 and 1992, followed by an interesting recovery in 1995 [13].

Comparing present data on metal concentrations with the results we obtained in the third campaign, testing Castel Giubileo, Ponte Tor di Quinto and Ponte della Magliana [13], some important differences can be pointed out: in the present campaign, concentrations of Cu and Cd are much lower than in the next campaign, for Ni and Zn a quite unchanged situation is recorded between 1992 and 1995.

Crude extracts of sediments sampled in the Venice lagoon characterised by a PAH concentration ranging from 65 to 460 ng/g dry matrix gave negative results to the Salmonella/microsome plate incorporation assay. Mutagenic effects were observed only in one sample characterised by a remarkably high PAH content (48 μg/g) [28]. Measured PAH concentrations in the present paper are much lower than that which induced observable mutagenic damage among sediments from the Venice lagoon. Although PAHs are known pro-mutagens, few investigations and contradictory results concerning plant sensitivity to PAH mutagenic effects have been obtained until now. This may be due to the activation of chemicals

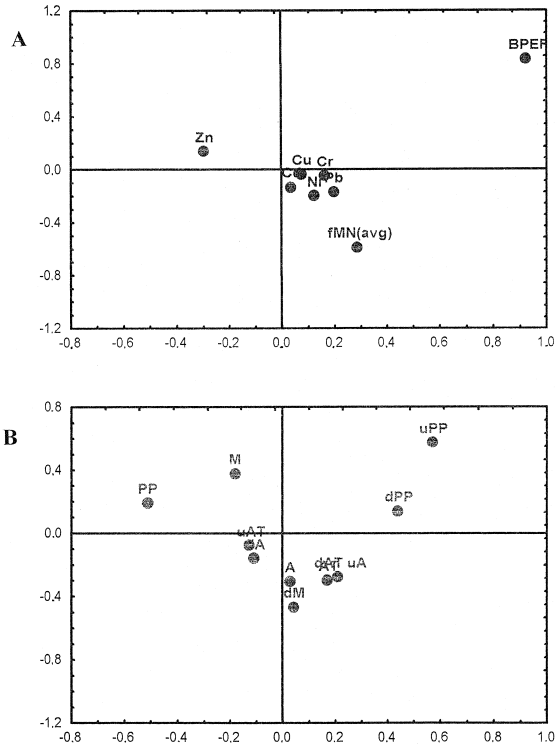

Fig. 3. Factorial correspondence analysis on both independent variables (metal and PAH concentrations) and the dependent one (average micronucleus frequency) measured in sediments from four tributaries (Prima Porta, Acqua Traversa, Aniene and Magliana) and from the Tiber river, up- and downstream the confluence of tributaries. Data should be presented in the same figure but this would not allow to distinguish a clear pattern due to a potential overlapping of symbols. Therefore, data are presented in two sets: in set A, both dependent and independent variables have been described; in set B, observations, i.e., sampling stations. (A) Variables. Points corresponding to metals are indicated by their symbols, i.e., Cd, Cr, Cu, Ni, Pb and Zn; the point corresponding to PAHs describes the pooled concentration values, expressed as toxicological Benzo(a)Pyrene Equivalent units (BPE units) of PAHs which have a well-known carcinogenic activity (see text) and it is indicated by BPE; the point corresponding to the average micronucleus frequency is indicated by fMN. (B) Sampling stations. Points corresponding to the sampling stations are indicated by their acronyms (see text: P.P., A.T., A., M, u.P.P., d.P.P., u.A.T., d.A.T./u.A., d.A., d.M.). Axis 1 is mainly determined (84%) by BPE and (negatively) Zn concentration; axis 2 is mainly determined (86%) by BPE concentration and (negatively) micronucleus frequency (fMN). The two main axes contribute to 81% of the total variance. It is noteworthy that points describing metals, with the exclusion of Zn, group towards the centre together with most of the stations (A.T., A., u.A.T., d.A.T./u.A., d.A., d.M.), whilst fMN lies a little a part.

to nonmutagenic quinones by different enzyme systems [29,30].

4.2. Pollutant concentrations and mutagenic effect

Multiple linear regression analysis shows a weak correlation among pollutant concentrations and micronucleus frequency ($r = 0.6$), being some regression coefficients negative (e.g., BPE: -0.17; Zn: -0.11) and other ones positive (e.g., Pb: 1.27; Ni: 1.7). As a possible explanation, the analysed pollutants are not the main responsible for the mutagenic effect, the cause of which must be searched elsewhere; however, it is also possible that other factors affected mutagenic action of pollutants (e.g., a different oxidative state of metals, a different bioavailability mediated by different solubility in water).

On the basis of Factorial Correspondence Analysis (Fig. 3), it is shown that the two main axes contribute to 81% of the total variance, suggesting a nonrandom distribution of points. Axis 1 is mainly determined (84%) by BPE and (negatively) Zn concentration; axis 2 is mainly determined (86%) by BPE concentration and (negatively) micronucleus frequency (fMN).

It is noteworthy that some stations are clustered together in a central position (uAT, AT, dAT/uA, A, dA, dM) and lie near metal concentrations (excluding Zn) and, at a lesser extent, micronucleus frequency; all these stations are downstream a long stretch of water courses flowing in highly urbanised areas, while all the other scattered stations collect waters which did not run in densely inhabited areas. In the former, the ratio between micronucleus frequency and metal concentration is higher, on the average, than in the latter (compare Tables 1 and 2); all these observations lead to the hypothesis that in sediments from ‘urbanised’ stations undetected co-pollutants are present which may increase the bioavailability of metals (e.g., chelating agents) and give, by this way, a higher yield in micronucleus induction.

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