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TOBACCO PLANTS DETECT A DECREASE OF ENVIRONMENTAL GENOTOXICITY IN TOULOUSE (FRANCE)

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Abstract. Tobacco plants, heterozygous for two independent *loci* involved in the chlorophyll parenchyma differentiation, allow the genotoxic effects of the atmosphere of the industrial estate South of Toulouse to be estimated. Somatic spots of green cellular colonies on yellow-green background, were counted to calculate the cellular rates of reversion. Two experiments were carried out in 1981, and in 1997. A general decrease of genotoxic effects was observed. These observations were interpreted as being due to a general decrease of the air pollution evaluated by the development of the concentrations of three toxic gases before and after the implementation of cleanup devices. The results obtained demonstrate the efficiency of this bio-indicator, which is easy to use and capable of integrating, *in situ*, genotoxic variations throughout the duration of plants' growth.

Keywords: atmospheric pollution, bio-indicator, ecotoxicology, genetoxicity, tobacco, urban atmosphere

1. Introduction

The accelerated urbanisation of the second half of 20th century has increased and diversified the sources of atmospheric pollution: exhaust gas of motor vehicles, industrial discharges, collective and individual heating, etc.

In the 1990's a number of measures were taken in Toulouse to reduce gas discharges into the urban environment. A device was constructed to filter the smoke emitted by the domestic garbage incineration factory situated south-west of the city (October 1st, 1994); gas washing devices were installed in the most important factories of industrial estate south of Toulouse; lights which interrupted the traffic on the ring road were removed and the first electrically powered underground railway line brought into service. The monitoring of the air quality was improved by installing a surveillance network managed by the Observatoire Régional de l'Air en Midi-Pyrénées' (ORAMIP) and by the City of Toulouse. In addition, studies of biological effects were performed independently for several years with the heterozygous genetic system of tobacco notably in 1981 (Devaud *et al*., 1984). With the

aim of estimating the repercussions of the work carried out to reduce the discharges of gas pollutants in Toulouse in recent years, an experiment using the same bio-indicator placed on the same sites was performed in 1997. Results and comparative analyses of these two studies are given in this document.

2. Materials and Methods

2.1. The test system

The bio-indicator used was the $a_1^+/a_1\ a_2^+/a_2$ system of *Nicotiana tabacum* var. *xanthi* n.c. (Dulieu *et al.*, 1971). It makes use of the properties of two *loci* heterozygous for mutant and wild type alleles, which lead to a slight chlorophyll deficiency (Dulieu *et al.*, 1971; Dulieu, 1974; Dulieu, 1975; Deshayes and Dulieu, 1974; Dulieu and Dalebroux, 1976), giving a yellow-green colour to the plants. Genetic reversions can be observed either spontaneously, or due to effect of a mutagenic agent and correspond to allelic reversions of a_1 towards a_1^+, of a_2 towards a_2^+ or of deletions of the gene a_1. In consequence, the modified cell increases its chlorophyll synthesis capacity, divides in a growing leaf and passes its new genotype and its colour to its cellular lineage. After several divisions, this can be detected in the palissadic parenchyma as a green spot.

This heterozygous structure reveals the genetic effect of low or very low doses of natural (Delpoux and Dalebroux, 1981a; Delpoux and Dalebroux, 1981b; Delpoux and Dalebroux, 1981c; Delpoux *et al.*, 1997) and artificial (Dulieu and Dalebroux, 1975; Fabries and Delpoux, 1978) radioactivity, cosmic radiations (Bayonove *et al.*, 1984), and the polluted air in various urban or industrial sites (Devaud *et al.*, 1984).

2.2. Calculation of the reversion rate

The experiment lasted for two months (August and September) with monthly measurements on leaves that had completely developed on the site under study. At the end of August all plants were harvested and analysed and were replaced by others which were harvested at the end of September. The genetic effects were calculated as average reversion rate (r.r.) per cell cycle. It has been shown (Dulieu and Dalebroux, 1975; Fabries and Delpoux, 1978) that the reversion rate is equal to:$r.r. = 1 - [(S - Sg)/S]^{1/t}$ where S and Sg are the total and reverted leaf areas of the individual examined, and t the number of cells cycles that actually took place during experiment: $t = (logN/log2) - 7$ N is the total number of cells observed related to the total leaf area S with cell density (d); N was estimated by $N = d\mathrm{x}S$. The number of 7 cellular generations was removed to be able to neglect spots having an area too small to be visible to the naked eye (less than 2^7 cells).

2.3. Standardisation of values

To compare results between the experiments of 1981 (Devaud *et al.*, 1984) and those of 1997 the values had to be standardized. Indeed, as has been noticed in the past, seed groups used at different times exhibit different spontaneous reversion rates (Delpoux and Dalebroux, 1981a; Delpoux and Dalebroux, 1981b; Delpoux and Dalebroux, 1981c; Fabries and Delpoux, 1978). This is due to the:

– biodiversity among plants used to produce the seeds for the experiments;
– variability within a given seed group, due to various factors and, in particular, the duration of storage.

This standardisation was made on the basis of values obtained in 1997 (August and September) at a control station (station 1 – *cf.* 'Network' and Figure 1).

2.4. Analysis of genotoxic effects

Five groups of three plants in 1997 and seven groups of three plants in 1981 (*cf.* Table I) were analysed for each monitoring station. Analysis of variance was applied to mean reversion rates per group. (*cf.* Table II). Pair-wise comparisons of means were made using the Dunnett test (Dunnett, 1955; 1964; 1980) ('one sided') with the control station or zone for reference value. The standard errors of each comparison were calculated using the mean square error. Mean values per station, per year and per month, are presented in Figure 2 where the statistically significant differences at $P_{0.5}$ are symbolised.

2.5. Station and plant culture

The seeds were sown on a moistened sterilised leaf mould in a greenhouse. When the seedlings had reached a height of 2 to 5 cm, they were individually transplanted into 13-cm-diameter pots containing sterilised leaf mould (2/3) and vermiculite (1/3). Twenty tobacco plants in the stage 5 to 6 leaves were installed in each station. The station consisted of a wire fence doubled by a windbreak of height equal to that of plants at the end of growth and covered with split cane to avoid direct sunshine on the photosensitive, yellow-green plants. The ground was a 10-cm layer of sand on a plastic film raised at the edges, allowing a suitable and constant humidity to be maintained between waterings to avoid any hydric stress. Cultivation methods were the same for all stations: when a phytosanitary product was needed, even locally, it was applied to all plants of all stations; water used was of the same origin for all stations.

2.6. Monitoring network

Stations were established at the following sites, indicated on Figure 1: Station 1 was located 12 km north-east of the centre of Toulouse in a rural environment at an altitude of 145 m on the north-east hillside of the Girou valley. Due to this position,

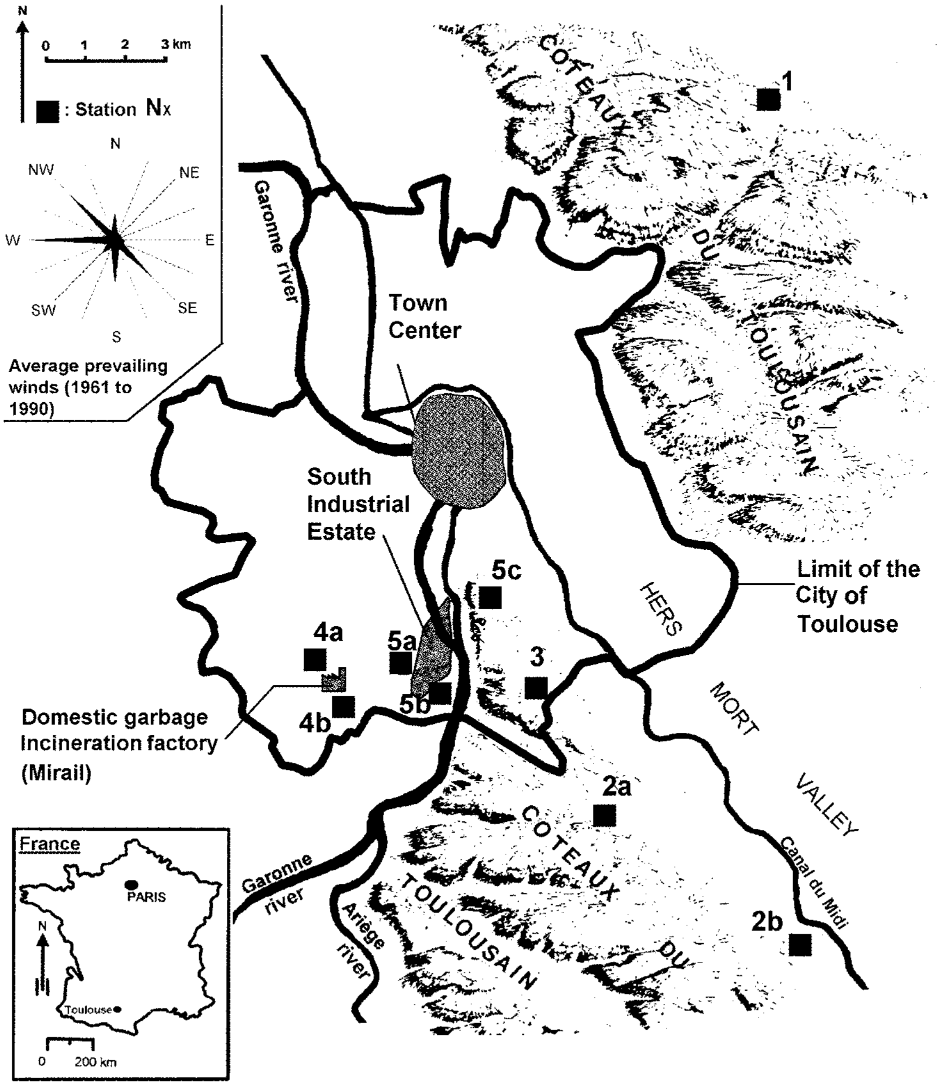

Figure 1. Location of the experimental stations with regard to the geographic and geomorphologic regional contexts.

and in view of dominant winds (Figure 1) established by Toulouse-Blagnac meteorological station, this site is not influenced by air released from the conglomeration and was used as the reference station. Stations 2a, b were located south-east of Toulouse in the direction of dominant West to Northwest winds. Station 3 was also located to the south-east of Toulouse, but nearer to the conglomeration than stations 2a and 2b. Stations 4a and 4b were, respectively, situated north-west and south-east of a domestic garbage incineration factory. They were then, respectively,

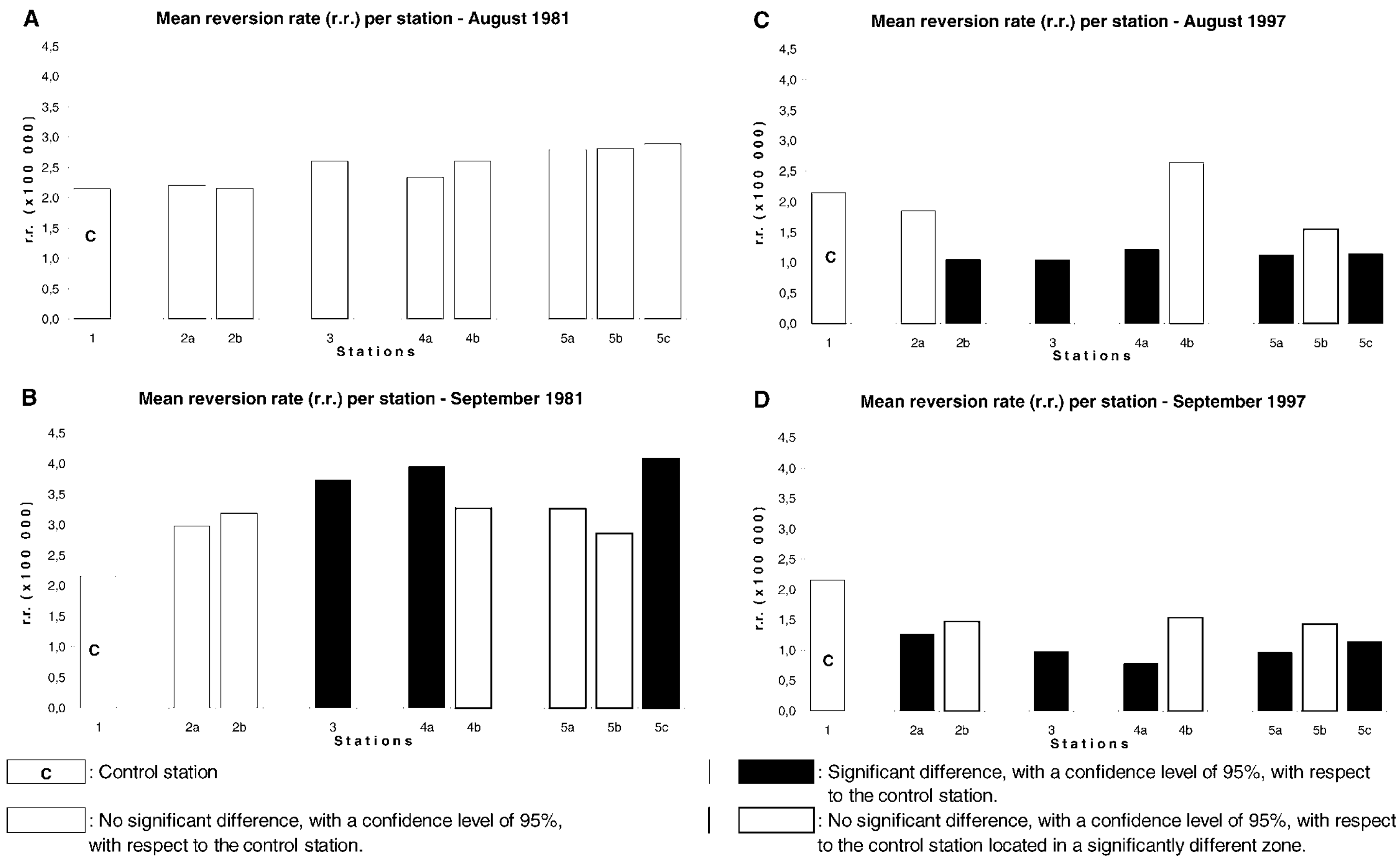

Figure 2. Mean reversion rates obtained in Toulouse and the rural neighbourhood during August and September, 1981 and 1997.

TABLE I

Mean reversion rates by groups and stations for the 1981 and 1997 experiments

			Year 1981									Year 1997						
Per- iods	Zo- nes	Sta- tions	N	Reversion rate per groups 1	2	3	4	5	6	7	M	N	Reversion rate per groups 1	2	3	4	5	M
	1	1	7	2.97	2.28	1.72	1.53	2.33	2.13	2.12	2.15	5	1.22	2.66	3.01	2.25	1.63	2.15
A	2	2a	7	3.43	2.04	1.79	1.65	2.70	1.85	2.00	2.21	5	1.55	2.42	1.22	1.30	2.73	1.84
U		2b	7	2.10	2.55	1.81	2.81	3.04	1.27	1.68	2.16	5	0.96	1.86	1.41	0.58	0.43	1.05
G	3	3	7	2.25	2.79	1.45	2.26	3.45	2.82	3.19	2.60	5	1.43	1.18	0.98	0.48	1.13	1.04
U	4	4a	7	2.01	2.36	3.38	2.07	1.84	3.04	1.69	2.34	5	1.34	1.11	1.27	0.93	1.43	1.22
S		4b	7	2.26	3.46	2.06	3.04	1.90	2.99	2.47	2.60	5	2.46	2.81	3.19	2.31	2.44	2.64
T		5a	7	2.62	1.55	2.45	2.77	2.38	5.34	2.47	2.79	5	1.06	0.74	0.76	1.38	1.71	1.13
	5	5b	7	3.26	3.21	2.28	2.56	3.80	2.21	2.40	2.82	5	1.33	1.46	1.52	2.13	1.31	1.55
		5c	7	3.20	2.28	3.76	4.66	1.81	2.78	1.76	2.89	5	1.19	1.26	1.42	0.89	0.95	1.14
		Mean									2.51							1.53
S	1	1	7	2.01	1.90	1.78	1.81	2.00	3.41	2.18	2.16	5	2.75	1.59	1.86	2.73	1.86	2.16
E	2	2a	7	3.08	1.96	4.02	3.06	2.77	4.45	1.52	2.98	5	0.90	1.54	1.13	1.67	1.07	1.26
P		2b	7	1.79	3.49	3.24	3.34	4.15	3.16	3.10	3.18	5	1.69	0.67	1.82	0.82	2.35	1.47
T	3	3	7	4.40	3.56	2.66	3.09	3.28	6.17	2.96	3.73	5	0.86	1.20	0.74	1.09	0.99	0.98
E	4	4a	7	2.41	4.17	6.84	2.72	3.65	2.62	5.27	3.96	5	0.50	1.17	0.54	0.81	0.90	0.78
M		4b	7	2.72	1.53	3.19	1.95	3.59	6.18	3.74	3.27	5	2.29	1.73	1.57	0.81	1.27	1.53
B		5a	7	4.18	1.96	4.16	1.82	4.18	2.54	3.97	3.26	5	1.11	0.98	0.71	1.29	0.73	0.97
E	5	5b	7	3.91	1.90	2.01	3.71	2.90	3.20	2.34	2.86	5	1.38	1.39	2.26	1.29	0.84	1.43
R		5c	7	6.61	3.59	3.54	1.56	5.10	4.67	3.48	4.09	5	0.77	1.88	1.14	1.19	0.73	1.14
		Mean									3.28							1.30

M: Mean reversion rates per station

N: number of groups per station.

in the direction of the south-easterly and the north-westerly winds (see prevailing wind, Figure 1). Station 5a was located west of the industrial estate. Station 5b was situated South and station 5c, Northeast of the same zone.

3. Results

3.1. Analysis of genotoxic effects

Experiment made in 1981:

Variance analysis (Table II) showed a significant effect from ‘Months’ and ‘Stations’, and a non-significant interaction ‘Month * Stations’. A comparison of mean rates confirmed a significant increase between August and September: 2.51 and 3.28, respectively. For September, the Dunnett tests indicated significant differences between mean rates, differentiating ‘Zones’ and ‘Stations’ (Figure 2, B).

TABLE II

Analysis of variance of the results of the experiments

Source	SS	dF	MS	F-ratio	
Experiment 1 (1981)					
treatments	43.1333	17	2.5372	2.56	**
months	18.3773	1	18.3773	18.55	***
stations	16.705	8	2.0881	2.11	*
interaction	8.051	8	1.0064	1.02	ns
error	106.9714	108	0.9905	–	
Experiment 2 (1997)					
treatments	20.438	17	1.2022	5.46	**
months	1.1606	1	1.1606	5.27	*
stations	15.9899	8	1.9987	9.08	**
interaction	3.2875	8	0.4109	1.87	ns
error	15.8496	72	0.2201	–	
August 81 + 97					
treatments	43.124	17	2.5367	5.89	**
years	25.2334	1	25.2334	58.58	***
stations	6.4571	8	0.8071	1.87	ns
interaction	11.4335	8	1.4292	3.32	**
error	38.769	90	0.4308	–	
September 81 + 97					
treatments	128.6513	17	7.5677	8.15	**
years	102.0149	1	102.0149	109.88	***
stations	4.8399	8	0.605	0.65	ns
interaction	21.7965	8	2.7246	2.93	**
error	83.5585	90	0.9284	–	

Indeed, stations 3, 4a, and 5c had significantly higher means in September than the control station (Table I). This was also true for the zones 3, 4 and 5 (Figure 2, B).

Experiment made in 1997:

Variance analysis (Table II) showed a significant effect from 'Months' and 'Stations'. The interaction 'Months * Stations' was not significant. The comparison of mean rates (Table I and Figure 2) revealed a decrease in reversion rates in August and September, compared to the control site (station 1), except for 4b in August.

TABLE III

Annual average concentrations of SO_2, NO_2 and HCl

SO_2	Years	–	–	–	1992	1993	1994	1995	1996	1997	1998	1999
	Concentrations (μg m^{-3})				16	21	13	15	9	8	9	8
NO_2	Years	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999
	Concentrations (μg m^{-3})	186	196	196	188	109	91	99	94	87	86	90
HCl	Years	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999
	Concentrations (μg m^{-3})	11	14	18	12	10	11	3	3	3	4	3

–: no measurements available.

TABLE IV

Measurement of three main pollutants (SO_2, NO_2 and HCl) on a monthly basis

Mois	SO_2 – Average contents		Monthtly averages NO_2 P98		HCl – Averages of monthly maxima	
	1992 to 1994	1995 to 1997	1992 to 1993	1995 to 1997	1992 to 1994	1995 to 1997
Jan.	30.0	13.3	93.0	54.0	8.2	3.1
Feb.	21.5	16.3	94.0	50.5	9.4	3.1
March	19.0	15.7	198.5	57.5	12.6	4.3
April	17.5	12.7	71.5	47.0	11.7	3.8
May	17.5	9.0	53.0	39.0	20.6	2.3
June	17.3	11.3	89.0	43.5	13.9	1.9
July	14.0	9.3	72.5	44.5	6.7	2.7
Aug.	13.0	8.7	55.0	39.0	7.7	1.7
Sept.	20.0	12.7	74.5	55.0	7.2	3.3
Oct.	16.0	11.7	51.5	57.5	6.4	2.5
Nov.	17.0	13.7	74.5	59.5	8.1	2.7
Dec.	22.5	12.7	91.0	51.5	11.7	4.7

Both experiments 1981 and 1991, analysed together:
Variance analysis for August (1981 + 1997) showed that all effects, except 'Stations', were significant. The Dunnett test confirmed that the rates decreased significantly from 2.51 to 1.53 between 1981 and 1997,.

Variance analysis performed for September (1981 + 1997) showed that all the effects were significant, except 'Stations'. The Dunnett test confirmed that the rates observed in September decreased strongly compared to August, from 3.28 to 1.30.

3.2. Analysis of the air

The urban and industrial sites showed a reduction in primary atmospheric pollutants between 1991 and 1999. The evolution of three main pollutants (SO_2, measured on the urban traffic site; NO_2 and HCl, measured near factories rejecting these gases) demonstrate this decrease (Tables III and IV).

3.2.1. *Sulphur dioxide*
The annual average concentrations of SO_2 from 1992 to 1999 showed a significant and regular decrease. While seasonal variations were still obvious (*cf.* Table IV), concentrations were always lower for comparable months.

3.2.2. *Nitrogen dioxide*
The annual average concentrations of NO_2 from 1989 to 1999 indicated a large decrease in 1993 doubtless related to the installation of a gas washing device in the factory 'Grande Paroisse', in the south of the industrial estate. The decrease is particularly significant for the winter months (*cf.* Table IV), moreover the amplitude between extreme values is also reduced (from 57 to 20.5 μg m^{-3}.

3.2.3. *Hydrochloric acid*
Table III shows the annual average concentrations of HCl from 1989 to 1999. The considerable decrease observed in 1995 also followed the installation of the gas washing device, (October, 1994). The averages of the monthly maxima (Table IV) are two to nine times lower in 1994 and the differences between maximal and minimal values was reduced to below 5 μg m^{-3}.

4. Discussion

In 1981, a significant increase of the reversion rates was observed between August and September for most of the stations (except the control and station 5b) in keeping with the seasonal change of socio-economic activity. Indeed, August corresponds to a period during which industrial activity and motor traffic are low. Furthermore, it is traditionally the month of holidays for most people in Toulouse and for nearly all students who represent a quarter of its population.

In 1997, regardless of the month considered, a marked falling trend in the reversion rates measured in the industrial estate and in rural zones to the south was noted with regard to the reference zone.

The assessment of pollution readily explains these results. Indeed, between 1981 and 1997, numerous atmospheric pollutants appreciably decreased. The annual average concentrations of SO_2 decreased by half between 1992 and 1997 on the sites of heavy traffic (16 μg m^{-3} and 8 μg m^{-3} respectively) and pollution measured in industrial areas also diminished. Because of the installation of gas washing devices in the most important factories, the annual average concentration of NO_2 was reduced by one half between 1989 (186 μg m^{-3}) and 1997 (87 μg m^{-3}) and that of HCl by 3.6 from 1989 (11 μg m^{-3}) to 1997 (3μg m^{-3}).

Furthermore, this decrease might also be explained by the much greater plant biomass in the new parks and leisure areas in 1997, notably the 'green' zones (stations 3 and 5a, 5b and 5c). In station 5b, it is interesting to note that much vegetation was already present in 1981 and this is the only station at a potentially

polluted site for which the increase of the average reversion rates was very small: 2.82 in August and 2.86 in September. In 1997, the negative differences between tested stations and the control could result from a pollution affecting the control station.

These results tend to indicate the efficiency of measures taken by the authorities and by certain manufacturers to limit gas discharges. They confirm the interest of the bio-indicator used to study atmospheric pollution and also suggest that more information could be obtained if a double network of chemical and biological surveillance of the air quality in urban zones were set up.

Acknowledgements

We thank very particularly Mr Alain Séverac, engineer at Paul Sabatier University of Toulouse for the active participation in the construction of the experimental stations and in the maintenance and surveillance of the Tobacco plant cultures.

We also thank Father Arnal, Priest of Pouvourville; Mr Dominique Baudis, Mayor of Toulouse; Mr Esparza, Engineer in the 'Compagnie Générale des Eaux' (C.G.E); Mr Fohano, farmer; Mr Péréa, manager; Mr Ponthus, Engineer at the 'Service Environnement' of the City of Toulouse; Mr Toulze, nursery gardener, and Mr and Mrs Vialar, householders in Mervilla, whose help and permission allowed us to install the network.

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