Possible Relevance of Pigeons as an Indicator Species for Monitoring Air Pollution

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Wild city pigeons were caught at four different locations in the Netherlands to represent areas of high (Amsterdam-high), moderate (Amsterdam-medium), and low (Maastricht and Assen) traffic density. It is assumed that local ambient air pollution decreases as a function of traffic density. In these pigeons levels of polycyclic aromatic hydrocarbons (PAH–DNA adducts, oxidative DNA damage, and heavy metal residues were determined in kidney, lung, liver, and blood (no adduct analysis in blood). The contribution of leaded gasoline to total body lead content was estimated by measuring concentrations of Pb and its isotopes in blood. We also analyzed samples of ambient air particulate matter for PAH and heavy metal concentrations at the four different locations. Interregional differences in heavy metals in ambient air particulate matter were reflected relatively well by pigeon body loads. The highest lead and cadmium concentrations in blood, kidney, liver, and lung were found in the Amsterdam-high traffic density area, followed by Amsterdam-medium, Assen, and Maastricht. A high Pb concentration in blood coincided with relatively low 206Pb/207Pb values, indicating a high contribution of leaded gasoline to total blood Pb concentrations in pigeons from the Amsterdam high traffic density area. Significantly enhanced blood zinc values were found in pigeons from both locations in Amsterdam compared to pigeons from the other two areas. However, no differences in Zn tissue levels between the four different groups were found. Oxidative DNA damage, determined as the ratio of 7-Hydroxy-8-oxo-2′-deoxyguanosine/deoxyguanosine, in pigeon liver was highest in Amsterdam-high, followed by Assen (low traffic density). Pb content, but not the Cd content, was positively associated with oxidative DNA damage in liver tissue. In lung tissue, a negative correlation was found between oxidative DNA damage and Zn content. These results indicate that the carcinogenic potential of Pb might be ascribed to oxygen radical formation, whereas Zn plays a protective role against oxidative DNA damage. Places with high and medium traffic density could be clearly discriminated on the basis of PAH levels in the ambient air. The PAH content in particulate air samples was not, however, reflected in total PAH-related DNA adduct levels because no differences could be observed in tissue adduct levels in pigeons from the four different locations. Our results indicate that wild city pigeons can be used as biological indicators of exposure to heavy metal pollution in outdoor air. Key words: air pollution, cadmium, lead, lead isotopes, oxidative DNA damage, PAH–DNA adducts, pigeons, zinc.

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The increase in industrial activities and traffic in the past century has caused severe air pollution (1). In urban areas automobile exhaust constituents significantly contribute to air pollution. Vehicle discharges include carbon monoxide, uncombusted hydrocarbons, nitrogen oxides, volatile organic substances such as benzene and toluene, and heavy metals such as lead (Pb) (2,3). Epidemiological studies have shown an increase in respiratory diseases such as bronchitis and an increase in lung cancer incidence in urban areas as compared to rural areas (2,4,5). Concern for the potentially mutagenic and carcinogenic effects of air pollutants has therefore resulted in numerous studies of the mutagenic activity of extracts of particles of airborne pollutants (6–8). Less attention has been paid to the ecological impact of these air pollutants. It is generally difficult to find suitable biological indicators for areas with high traffic density (9). In a few studies pigeons have been used as biological indicators of the Pb concentration in the ambient environment (9–13). Pigeons have a rather small habitat, a small body size, a high metabolic turnover, and a high inhalation rate and might therefore be used as a biological indicator for ambient air pollution. The main routes of exposure include inhalation of contaminated air and ingestion of food and soil particles contaminated by deposited air pollutants. The carcinogenicity of ambient airborne particles is reported to be associated largely with the presence of polycyclic aromatic hydrocarbons (PAHs) (2,3,14). It has been suggested that the extent of binding of carcinogenic xenobiotics, such as metabolites of PAHs, to DNA in organisms may serve as a dosimeter at the target site. Determination of PAH–DNA adducts has been successfully applied by means of 32P postlabeling in environmentally exposed humans (15,16), fish (17–20), earthworms (21), mussels (22), and plants (23). Moreover, wild woodchucks living in PAH-contaminated areas exhibited higher benzo[a]pyrene–protein adducts in blood compared to animals living in control areas (24). Determination of PAH–DNA adduct formation in tissues of wild city pigeons may therefore provide biologically relevant information on the carcinogenic potency of the contaminated inhaled air.

In addition to organic pollutants, automobile exhaust constituents include heavy metals such as Pb. Unlike organic compounds, metals are not biodegradable and accumulate in environmental compartments and in organisms. Several reports indicate that 90% of the total atmospheric Pb emissions are derived from automobile emissions (1,25,26). There are different possible sources of Pb exposure such as gasoline, atmospheric dust, street dust, paint, and natural sources. Pb has the advantage of having stable isotopes in different abundances, depending on its geochemical history, which in certain cases enables tracing of the origin of Pb in the environment. Pb has one nonradioactive isotope, 206Pb. The other isotopes, 205Pb, 207Pb, and 208Pb, result from the decay of 238U, 235U, and 232Th, respectively. The affect of traffic can be determined by measuring Pb concentrations and isotopes. Pb in gasoline in The Netherlands is a mixture of Pb from Australian and Canadian ores. The average 206Pb/207Pb values for gasoline are taken from 10 different Scottish leaded samples (206Pb/207Pb = 1.082) measured from 1989 to 1991 in brands that are also available in The Netherlands (27). Natural sources are assumed to have 206Pb/207Pb values ranging from 1.20 to 1.30, with an average 206Pb/207Pb of 1.25 (28). The best approximate 206Pb/207Pb composition of airborne dust that pigeons may be exposed to is the composition of

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atmospheric dust in Western Europe, which measures 1.130 (29). This value is only slightly higher than that for Pb in gasoline, indicating the large influence of automobile exhaust on the airborne dust composition. Street dust is expected to have a similar Pb composition as atmospheric dust (27) and represents the major exposure route to air pollutants via inhalation or ingestion of contaminated food particles. Using inductively coupled mass spectrometry (ICP-MS) to analyze blood (30–34), it is possible to trace the origins of Pb in pigeons exposed to contaminated air.

Other heavy metals significantly contribute to air pollution as well. Anthropogenic emissions of metals, for example cadmium (Cd) and zinc (Zn), into the environment have increased enormously since the industrial revolution. Nonferrous smelters and secondary production plants are the main sources (1).

Recent studies indicate oxidative stress as a causal factor in the adverse effects of organisms exposed to heavy metals (26). Oxidative stress may cause several types of DNA lesions. 7-Hydroxy-8-oxo-2'-deoxyguanosine (8-oxodG) can be detected in DNA hydrolysate by HPLC in combination with electrochemical detection (ECD) (35). This provides the possibility to investigate the relationship between oxidative DNA damage in tissues of pigeons and oxygen-radical–forming agents such as metals in contaminated air.

This study was conducted to determine the levels of PAH–DNA adducts, oxidative DNA damage, and heavy metal residues in tissues of wild city pigeons in Amsterdam (two locations with high and medium traffic density), Maastricht, and Assen, The Netherlands, with the assumption that air pollution decreases as a function of traffic density. Particular air samples were taken at four different locations and analyzed for PAH and metal concentrations. In this way the relevance of pigeon dosimetry as a biological indicator of urban air pollution was established.

Materials and Methods

Materials

Proteinase K (specific activity 20 U/mg lyophilisate), RNase A (specific activity 50 U/mg dry powder), RNase T1 (specific activity 100,000 U/ml), and α-amylase (specific activity 1,000 U/mg) were purchased from Boehringer Mannheim (Mannheim, Germany). Alkaline phosphatase (type VII-N; specific activity 10,000 U/ml), micrococcal nuclease (100–200 μM U/mg protein), potato apyrase (3–10 U/mg protein), and nuclease P1 (specific activity 200 U/mg protein) were obtained from Sigma (St. Louis, Missouri). Spleen phosphodiesterase (2 U/mg) was obtained from Cooper Biomedical Corp., T4 polynucleotide kinase (specific activity 30 U/μl) and γ-32P from Amersham, (Buckinghamshire, UK) and polyethyleneimine-cellulose TLC sheets from Machery-Nagel, (Düren, Germany). Methanol was HPLC grade and was obtained from Rathburn (Walkerburn, UK). Hydrogen peroxide, suprapur, and TI-thioglycolic acid standard were obtained from Merck (Amsterdam). SRM standards, Pb isotope and reference materials, were from the U.S. National Bureau of Standards. Analytical-grade chemicals were used for all other chemicals. Water was purified by means of a milli-Q water purification system.

Selection of Birds

A total of 29 wild city pigeons was caught at four different locations with varying traffic density. In Amsterdam, two feeding places were selected, one directly next to a main road (high traffic density) and one farther away from a main road (medium traffic density). In Maastricht and Assen, the traffic density was lower than in Amsterdam (no traffic lights or intersections). The birds were caught with trap cages.

The birds were sacrificed under CO2 anesthesia by decapitation immediately upon arrival. The organs were dissected, washed in ice-cold phosphate-buffered saline (pH 7.4), and after being weighed immediately frozen at -80°C until further analysis. The pigeon blood was collected in 50-ml sterile vials and refrigerated at -20°C.

Atomic Absorption Spectrometry

Samples of liver, lung, and kidney were dried at 120°C to a constant weight and subsequently ground to powder using an agate mortar. Each sample was treated with 20 ml of a 1:1 mixture of concentrated HNO3 and water and subsequently boiled for 2 hr on a hot plate. After filtration the filtrate was evaporated to a moist residue (2–3 ml) and subsequently diluted with 0.1% HNO3. Concentrations of Cd and Pb were measured by graphite furnace AAS with Zeeman background correction and concentrations of Zn by flame AAS with deuterium background correction. Pb, Cd, and Zn analysis were carried out at 283.3 nm, 228.8 nm, and 213.9 nm, respectively. The ashing temperature was 500°C for Pb and 500°C for Cd; the samples were atomized at 2,100°C and 1,800°C, respectively. Pb, Cd, and Zn-nitrate solutions in 0.1% HNO3 were used for calibration (external standard line). Paladium-nitrate was used in all instances as a modifier. All glassware was rinsed with 1% HNO3 to avoid contamination.

Air Sampling and Analysis

Environmental monitoring is conventionally performed by trapping the particulate bound fraction onto glass fiber filters using high-volume techniques (36,37). We have collected both total suspended particulate matter (TSP; particles with diameters between 0.1 and 10 μm) and PM10 (particles with a diameter <10 μm) on type wheel-conditioned glass fiber filters (Type A/E, Gelman Sciences, Ann Arbor, MI), which were weighed again after sampling and conditioning; the high-volume sampler was set at 1,100 l/min. For the determination of PM10 the high-volume sampler was equipped with a PM10, size-selective inlet; a cyclone. All four locations were sampled for 2 days; one day for PM10, one day for TSP, between 10:00 A.M. and 6:00 P.M., under comparable weather conditions. All samples were taken within a 1 month period to avoid seasonal influence on sampling. The filters were subsequently cut into two pieces; one half was used for analyses of Cd, Zn, and Pb; the other half for PAH content. The PAHs analyzed were the 16 PAHs identified as priority pollutants by the U.S. Environmental Protection Agency: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylenne, dibenzo[a]anthracene, and indeno[1,2,3-c-d]pyrene. Acenaphthylene cannot be measured by fluorescence detection (36).

For PAH analysis, filters were cut into tiny pieces, and these pieces were Soxhlet extracted with 120 ml petroleum ether for 16 hr, after which the extract volume was reduced by rotary evaporation. Part of this extract was dried under nitrogen to a small residue, which was subsequently dissolved in a known volume of acetonitrile. Samples were analyzed at 30°C by reverse-phase HPLC with fluorescence detection (Perkin Elmer LS30) using a 5 μm Vyde ODS column (250 × 4.6 mm). The mobile phase consisted of water and acetonitrile. Gradient elution started with 75% of mixture B (40% acetonitrile, 60% water; v/v) and 25% mixture A (100% acetonitrile). A linear gradient was used from 5 to 25 min resulting in 10% B and 90% A. The excitation wavelength was set at 250 nm while a cut-off filter of 350 nm was installed. The concentrations of particular PAHs were determined by comparing peak heights with those of known standards. Heavy metal content of particulate matter on the filters was determined according to NEN 6465 (38). In short, a glass fiber filter was cut into tiny pieces, and these pieces were
boiled under reflux for 2.5 hr in Aqua Regia and subsequently filtrated. Heavy metal analysis was performed as described above.

Inductively Coupled Plasma Mass Spectrometry
Duplicate blood samples (0.5 g) were digested with 2 ml HNO₃ (double sub-boiled) and 0.5 ml of hydrogen peroxide in 15 ml vials. After 24 hr of gentle, complete digestion at room temperature, the sample was heated to 80°C to boil off any excess hydrogen peroxide. Cooled samples were diluted to approximately 10 ml by weight. Samples were run in two runs (with samples in different order) with SRM981, SRM982, and SRM983 standards and blanks measured before and after every seven samples. Long-term reproducibility during the period of measurement was better than 1% for ²⁰⁶Pb/²⁰⁷Pb. Reproducibility of in-run triplicate samples (n = 5) was better than 0.5%. Mass bias correction for ²⁰⁶Pb/²⁰⁷Pb was applied based on the in-run SRM981 measurements (n = 6) for each duplicate run. Blanks contained <0.17 pg/g with variable isotopic ratios inferred to be the result from memory effects in the nebulizer rather than from sample contamination. Values for each run were averaged. Concentrations of Pb, Cd, and Zn in pigeon blood were analyzed in a separate run in the same solution. Measurements were performed on a VG Plasma Quad inductively coupled plasma mass spectrometer (Vacuum Generators, Manchester, UK) with thulium as internal standard.

Estimation of Lead from Gasoline
Estimation of the portion of Pb from gasoline present in pigeons was estimated using the following mass balance:

\[ R_{\text{measured}} = (R_{\text{gasoline}})X + R_{\text{other sources}}(1-X) \]

where \( R \) stands for the isotope ratio Pb / Pb (\( R_{\text{gasoline}} \) = 1.082, \( R_{\text{other sources}} \) = 1.25) and \( X \) is the fraction of gasoline Pb.

DNA Extraction and Digestion
DNA from liver, lung, and kidney tissue was isolated according to a standard procedure (39). Briefly, tissue samples (one kidney, one lung, and one liver lobe) were cut into pieces and homogenized in 1% SDS/1 mM EDTA and subsequently incubated with proteinase K. The tissue homogenate was successively extracted with 1 volume of phenol, 1 volume of phenol/chloroform/isoamylynol alcohol (25/24/1) and 1 volume of chloroform/isoamylynol alcohol (24/1). After DNA precipitation using 0.1 volume of sodium acetate and 2 volumes of ice-cold ethanol, DNA was dissolved in 5 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. RNA was destroyed by addition of RNase T1 and RNase A. Liver samples were subsequently treated with α-amylase to remove remaining glycogen. After extraction of the digest with 1 volume of chloroform/isoamylynol alcohol (24/1), DNA was precipitated and solubilized as described above. DNA concentration was assayed spectrophotometrically. The DNA was digested to deoxynucleosides by treatment with nuclease P1 (25 U/mg DNA) and alkaline phosphatase (25 U/mg DNA) (40).

HPLC/ECD Analysis of 8-oxodG
8-oxodG was quantitated by HPLC/ECD, which was performed using a Spectroflow 480 solvent delivery system (Separations, H.I. Ambacht, The Netherlands) coupled with a Kratos spectroflow 783 programmable absorbance detector (Separations) and an Antec electrochemical detector (850 mV) (Antec leyden bv, Leiden, The Netherlands). A Supelcosil column (Supelco; 250 x 4.6 mm) was used in conjunction with a guard column (ODS pellicular 30 x 2.1 mm). The mobile phase consisted of 15% aqueous methanol containing sodium acetate (25 mM), citric acid (12.5 mM), NaOH (30 mM), and acetic acid (10 mM). Elution was performed at a flow rate of 1.0 ml/min. The lower limit of detection was 40 fmol absolute for 8-oxodG; dG was simultaneously monitored at 260 nm. Oxidative DNA damage was expressed as the ratio of 8-oxodG to dG (number of adducts per 10⁶ dG nucleotides).

Determination of Aromatic DNA Adducts by [³²P]-Postlabeling
DNA samples were analyzed essentially as described by Reddy and Randerath (41). Five micrograms of DNA were digested for 3.5 hr at 37°C in the presence of 200 μM micrococcal nuclease and 2.8 μM spleen phosphodiesterase in a total volume of 5 μl containing 20 mM sodium succinate, and 10 mM CaCl₂, pH 6. Samples were then further digested with 6.25 μg nuclease P1 in a total volume of 10 μl, containing 0.03 mM ZnCl₂ and 3.75 mM sodium acetate pH 5, for 1 hr at 37°C. After the addition of 2.5 μl 0.5 M Tris base, the DNA digest was labeled with [³²P]ATP (50 μCi) using 5 U of T4 polynucleotide kinase. The [³²P]ATP was synthesized in the laboratory using carrier-free [³²P]Phosphate. The specific activity was determined as described and generally was 3,000–5,000 Ci/mmol (35). Purification and resolution of ³²P-labeled adducts was carried out on polyethyleneimine-cellulose TLC sheets. Chromatography was performed using the following solvents: D1, 1.0 M sodium phosphate, pH 6.0; D2, 3.5 M lithium formate, 8.5 M urea, pH 3.5; D3, 0.8 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 8.0; D4, 1.7 M sodium phosphate, pH 6.0.

The chromatograms were analyzed by a storage phosphor imaging technique (Molecular Dynamics, Sunnyvale, California). In each experiment [³²P]-benzo[a]pyrene-diol-epoxide (BPDE)—DNA standards with a modification level of 1 adduct in 10⁷, 10⁸, and 10⁹ nucleotides were included. Adduct levels were calculated from the amount of radioactivity on the chromatograms and

| Adduct-yielding PAHs | Fluoranthene | Benz[a]anthracene | Chrysenes | Benzo[b]fluoranthene | Benzo[k]fluoranthene | Benzo[a]pyrene | Dibenzo[a,l]anthracene | Indeno[1,2,3-cd]pyrene | Sum |
|---------------------|-------------|-----------------|----------|---------------------|---------------------|----------------|-----------------------|----------------------|------|
| µg dust/m²          | 88.4        | 63.1            | 80.9     | 100.6               | 66.8                | 75.6           | 72.7                  | 31.1                 |      |

| High traffic | Medium traffic | Maastricht | Assen |
|--------------|----------------|------------|-------|
| TSP PM₁₀   | TSP PM₁₀ | TSP PM₁₀ | TSP PM₁₀ |
| 2.895        | 3.237       | 3.149     | 2.731 |
| 5.842        | 5.066       | 2.788     | 3.507 |

Table 1. Data on TSP- and PM₁₀-bound PAHs and heavy metals at the four different locations (ng/m³ air)

| Metals | Cd   | Zn  | Pb   |
|--------|------|-----|------|
| µg/m³  | 0.57 | 107.8 | 37.1 |
| 0.31  | 77.5 | 63.6 | 63.6 |
| 0.35  | 108.9 | 33.5 | 33.5 |
| 1.05  | 109.5 | 36.0 | 36.0 |
| 0.76  | 119.3 | 24.7 | 24.7 |
| 0.76  | 118.3 | 25.7 | 25.7 |
| 0.64  | 148.4 | 25.8 | 25.8 |
| 0.91  | 40.9 | 12.4 |      |

Abbreviations: TSP, total suspended particulates; PM₁₀, particulate matter <10 μm diameter; PAHs, polycyclic aromatic hydrocarbons; EPA, U. S. Environmental Protection Agency.
the DNA amount and normalized against the [H]-BPDE-DNA standards with known modification levels. These external standards were run on separate TLC sheets.

**Statistical Methods**

Simple regression analysis was based on log-transformed data. Statistical evaluation of differences between the several groups with respect to adduct levels, heavy metal content, and organ weight was performed using the Kruskal-Wallis test. Multiple regression analysis was performed with the log-transformed data of DNA adducts as the dependent variables. The independent variables included organ weight and the heavy metal content of the tissue expressed as micrograms per gram wet weight. We considered p-values <0.05 as significant. Because of the limited number of measurements of air pollutants, no statistical tests were performed.

**Results**

Table 1 presents the concentrations of PAHs in the ambient air that are known to yield DNA adducts through the formation of reactive diol-epoxides (fluoranthene, benzo[a]anthracene, chrysene, benzo[k]- and benzo[ell]-fluoranthene, benzo[a]pyrene, dibenzo[a]anthracene and indeno[1,2,3-c,d]pyrene) (40), the sum of the 16 EPA-listed PAHs, as well as the concentrations of Pb, Cd, and Zn at the four different locations. At two locations, PM10 values were higher than TSP values measured 1 day later. However, the PM10 fraction was very low for the control area, Assen, compared to the three other locations, and a rather low TSP value was found for Maastricht. The sum of the PAHs known to produce DNA adducts was approximately two times higher in the high traffic density area of Amsterdam compared to the medium traffic density location in Amsterdam; remarkably lower levels were found in the two control areas, Maastricht and Assen. Comparable values were found for the sum of the 16 PAHs. Ambient air Pb levels were also enhanced in both locations of Amsterdam as compared to the two control areas. The Cd and Zn levels showed no apparent differences between the four areas.

In Table 2 data are presented on Pb, Cd, and Zn contents in kidney, liver, lung (microgram per gram wet weight), blood (nanogram per milliliter, and organ weight in wild city pigeons. The accumulation pattern of these metals in the four different areas was comparable. The highest Pb contents were found in the kidney; in liver and lung the Pb levels were two to three times lower. Cd predominantly accumulated in the kidney, followed by the liver; significantly lower levels were found in lung tissue. The accumulation of Zn in liver and kidney tissue was comparable; lung tissue contained approximately half of the amount of Zn found in liver and kidney.

Kruskal-Wallis analysis demonstrated highly significant regional differences in Pb content in liver, kidney, and lung as well as in blood (p<0.001). Highest Pb levels were found in Amsterdam, followed by Maastricht and Assen. The contrast in ambient air Pb concentrations between two locations within Amsterdam was also reflected in significant differences in organ Pb content (Table 2). Pb contents in blood and organ tissue were approximately five times higher in the high traffic density location of Amsterdam compared to the medium traffic density area. Regression analysis showed a significant correlation between blood Pb levels and Pb content in liver (p<0.0001; r = 0.852), kidney (p<0.0001; r = 0.898), and lung tissue (p<0.001; r = 0.569). Figure 1 shows both the correlation between Pb content in blood and kidney and the marked regional differences in the amount of Pb in these tissues among the areas of heavy, medium, and light traffic density.

The results in Figure 2 show a correlation between the total Pb concentration in blood and the isotopic Pb composition (r = 0.491). The samples from the high traffic density location in Amsterdam clearly have the highest Pb concentration (253 ± 182 ng/ml) with the lowest 206Pb/207Pb (1.147 ± 0.006) indicating the highest contribution of Pb from gasoline. Pigeons from the Amsterdam location with less intense traffic had lower Pb blood levels (73 ± 16 ng/ml) and higher 206Pb/207Pb-ratios (1.154 ± 0.007). One of the pigeons from Assen had close to 2 μg/ml Pb in its blood.

### Table 2. Data on lead, cadmium, and zinc concentrations in kidney, liver, lung (μg/g wet weight), and blood (ng/ml). 206Pb/207Pb ratio in blood and organ weight in wild city pigeons (means ± standard deviation)

|          | Amsterdam | Medium traffic | Maastricht | Assen | Kruskal-Wallis p value |
|----------|-----------|----------------|------------|-------|-----------------------|
| Pb       | n = 8     | n = 8          | n = 5      | n = 7 |                       |
| Kidney   | 2.44 ± 1.30 | 0.53 ± 0.15   | 0.30 ± 0.19 | 0.41 ± 0.18 | 0.0005 |
| Liver    | 1.21 ± 0.75 | 0.18 ± 0.06   | 0.13 ± 0.12 | 0.16 ± 0.09 | 0.001   |
| Lung     | 0.65 ± 0.37 | 0.47 ± 0.27   | 0.31 ± 0.13 | 0.25 ± 0.12 | 0.01    |
| Blood    | 253 ± 182 | 73.3 ± 18.6 | 33.8 ± 30.4 | 26.8 ± 17.9 | 0.0001  |
| Blood: 206Pb/207Pb | 1.147 ± 0.005 | 1.154 ± 0.007 | 1.168 ± 0.018 | 1.156 ± 0.009 | 0.01    |
| Cd       | n = 8     | n = 8          | n = 5      | n = 7 |                       |
| Kidney   | 2.73 ± 2.61 | 2.51 ± 2.80   | 0.67 ± 0.83 | 0.60 ± 1.14 | 0.05    |
| Liver    | 0.43 ± 0.29 | 0.53 ± 0.50   | 0.27 ± 0.30 | 0.13 ± 0.18 | 0.05    |
| Lung     | 0.02 ± 0.01 | 0.03 ± 0.01   | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.05    |
| Blood    | 5.24 ± 2.48 | 6.20 ± 2.96   | 7.58 ± 2.60 | 6.87 ± 2.00 | 0.001   |
| Zn       | n = 8     | n = 8          | n = 5      | n = 7 |                       |
| Kidney   | 30.2 ± 7.6 | 28.4 ± 11.3   | 28.9 ± 12.7 | 20.7 ± 4.4  |
| Liver    | 35.8 ± 6.5 | 35.3 ± 8.9    | 31.2 ± 11.9 | 68.6 ± 65.1 |
| Lung     | 12.9 ± 5.2 | 16.2 ± 16.2   | 11.3 ± 7.4  | 8.4 ± 4.5   |
| Blood    | 1680 ± 100 | 2212 ± 289    | 1396 ± 282 | 1443 ± 401 | 0.001   |

**Figure 1.** Correlation between Pb concentrations in blood (ng/ml) and kidney (ng/g, wet weight) of wild city pigeons from Amsterdam-high, Amsterdam-medium, Assen, and Maastricht. Regression analysis; p<0.0001; r = 0.898.

**Figure 2.** Correlation between Pb concentrations in blood (ng/ml) and 206Pb/207Pb ratios of wild city pigeons from Amsterdam-high, Amsterdam-medium, Assen, and Maastricht. Regression analysis; p<0.001; r = 0.491.
which we assume was due to Pb shot (the data from this pigeon are excluded in all cases). The average Pb blood content of the other pigeons from Assen was 27 ± 18 ng/ml with an average 206Pb/207Pb ratio of 1.156 ± 0.009. The Maastricht pigeons showed a larger spread in values (Pb: 34 ± 30 ng/ml, 206Pb/207Pb: 1.168 ± 0.018), most likely due to the smaller amounts of pigeons analyzed in combination with higher analytical uncertainty because of the low concentrations.

Pigeons from the high traffic density location in Amsterdam had the highest percentage of Pb from gasoline (61.5%), followed by the medium traffic density location in Amsterdam (57.4%). Pigeons in Assen and Maastricht had 56.2 and 48.9%, respectively, Pb derived from gasoline in their blood (Fig. 3).

There was a significant difference in Cd content in all the organs examined between the four different locations (Table 2). Pigeons caught in Amsterdam accumulated more Cd in liver and kidney than pigeons from the control areas of Maastricht and Assen. No differences were found between the four different areas in blood Cd levels.

Analysis of Zn content in blood (Table 2) revealed a significant difference between Amsterdam and the two control areas in Zn levels in blood (Kruskal-Wallis: p<0.001). This was not, however, reflected in differences in Zn tissue levels.

The liver weight of pigeons from the light traffic density locations, Maastricht and Assen, was significantly higher than the liver weight of pigeons originating from Amsterdam (Table 2).

Data on adduct levels in liver, kidney, and lung are presented in Table 3. Oxidative DNA damage, determined as the ratio of 8-oxodG/dG, in pigeon liver was highest in pigeons from the Amsterdam-high traffic density area (p<0.05), followed by Assen. No significant differences in 8-oxodG levels of kidney DNA were observed in pigeons from the four different locations. Analysis of lung DNA revealed a significant increase in oxidative DNA damage in Maastricht compared to the other regions (p<0.001).

Regression analysis showed a positive significant correlation between Pb exposure and oxidative DNA damage in liver tissue (p<0.005; r = 0.538) (Fig. 4). In kidney and lung tissue, no correlation was found between Pb content and oxidative DNA damage.

In lung tissue, oxidative DNA damage appeared to be negatively correlated with Zn content of the lung (p<0.05; r = 0.341). Regression analysis within the individual groups revealed no significant correlation between those two parameters. A similar, although not significant, negative correlation between Zn levels and oxidative damage was found for the kidney.

No correlations were found between Cd levels in liver, lung, or kidney and oxidative DNA damage.

The autoradiograms of the 32P-labeled DNA digests showed diagonal radioactive zones (DRZs), which is typical for DNA adducts containing aromatic or bulky hydrophobic moieties (Fig. 5). The DRZs suggest the presence of multiple chemically related adducts. In kidney and liver of birds of all regions, there was an indication of increased density within the DRZ which represents major adducts. The nature of these putative adducts is unknown, but one co- chromatographed with the major DNA adduct formed by the reactive benzo[a]-pyrene (BaP) metabolite BPDE and DNA (BPDE-deoxyguanine) (Fig. 5D). The DRZ observed in lung was more diffuse.

The mean levels of aromatic DNA adducts per area are listed in Table 3. No clear differences in total DNA adduct levels, as reflected by the DRZ, could be observed between the different locations. There is only an indication that the total DNA adduct levels in the lungs of pigeons from the high traffic area in Amsterdam are somewhat higher compared with the DNA adduct levels in the lungs of pigeons from other locations.

### Discussion

In urban areas, traffic exhaust is one of the major sources of air pollution that may have adverse effects on human health and ecosystems (3,43). Automobile exhaust constituents include heavy metals such as Pb, carbon monoxide, uncombusted hydrocarbons, and several volatile organic substances. During the last decades concern about the hazards of toxic metals and environmental carcinogens such as BaP has increased worldwide.

Birds have been used previously as biological indicators of urban Pb pollution, demonstrating differences among areas of heavy and light traffic density (9–13). Pigeons may be especially useful because they can integrate body load by pollutants over time in an area-bound manner.

Atmospheric pollution by automobile exhaust also results in contamination of other environmental compartments (e.g., water, soil, and food). The total body load of pollutants in pigeons, therefore, is the

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Table 3. Data on oxidative DNA adducts and polyaromatic hydrocarbon-related DNA adducts in kidney, liver, and lung in wild city pigeons (mean ± standard deviation)

|          | Amsterdam | Medium traffic | Maastricht | Assen | Kruskal-Wallis p value |
|----------|-----------|----------------|------------|-------|-----------------------|
| 8-oxodG/dG (10^-6) |           |                |            |       |                       |
| Kidney   | 18.9 ± 9.34 | 17.4 ± 1.54 | 21.2 ± 2.5 | 22.8 ± 7.9 | 0.05 |
| Liver    | 2.86 ± 0.80 | 1.40 ± 0.56  | 1.72 ± 0.72 | 2.25 ± 1.30 | 0.05 |
| Lung     | 3.66 ± 0.66 | 5.22 ± 1.67  | 11.90 ± 2.32 | 7.52 ± 2.64 | 0.001 |
| BaP-related adducts (per 10^9 nucleotides) | |                |            |       |                       |
| Kidney   | 19.51 ± 12.96 | 3.53 ± 2.63 | 6.83 ± 5.22 | 26.93 ± 31.60 | 0.05 |
| Liver    | 9.21 ± 2.27 | 11.13 ± 5.65 | 19.33 ± 7.47 | 8.88 ± 7.29 | 0.05 |
| Lung     | 0.06 ± 1.08 | nd           | 0.33 ± 0.75 | 6.13 ± 13.14 | 0.05 |
| Adducts  (per 10^9 nucleotides) | |                |            |       |                       |
| Kidney   | 13.53 ± 9.10 | 12.45 ± 9.75 | 11.44 ± 6.75 | 10.08 ± 7.92 | 0.05 |
| Liver    | 10.47 ± 5.81 | 7.74 ± 3.61 | 12.04 ± 3.18 | 7.99 ± 3.58 | 0.05 |
| Lung     | 3.38 ± 2.51 | 3.58 ± 5.93 | 1.01 ± 0.40 | 4.02 ± 6.56 | 0.05 |
| DRZ adducts (per 10^9 nucleotides) | |                |            |       |                       |
| Kidney   | 14.33 ± 5.34 | 11.03 ± 5.49 | 12.26 ± 5.71 | 12.05 ± 5.35 | 0.05 |
| Liver    | 12.55 ± 4.03 | 11.55 ± 4.81 | 16.11 ± 5.17 | 11.83 ± 5.53 | 0.05 |
| Lung     | 5.62 ± 3.46 | 4.08 ± 3.22 | 4.50 ± 1.26 | 4.48 ± 3.72 | 0.05 |

Abbreviations: 8-oxodG/dG, 7-hydro-8-oxo-2'-deoxyguanosine/2'-deoxyguanosine; BaP, benzo[a]pyrene; DRZ, diagonal radioactive zone; nd: not detectable.
result of both inhalation of air pollutants and absorption of food and soils contaminated by deposited air pollutants.

Characterization of air quality in the Netherlands is largely conducted by the National Air Quality Monitoring Network at four measurement stations, representing rural, rural and industrial, urban, and urban and industrial settings. The data from these measurement stations are considered representative of comparable settings. The annual mean concentrations in outside air in The Netherlands for Cd varied in 1993 between 0.29 and 0.63 ng/m³, for Zn between 46 and 74 ng/m³, and for Pb between 18 and 59 ng/m³. Monitoring of PAHs in air, for which BaP is used as indicator, does not take place routinely. Incidental data on BaP in air vary from 0.2 to 0.5 ng/m³ (but this probably overestimates the average situation in The Netherlands).

Samples of ambient air pollution were taken at the four different locations and analyzed for PAH and metal concentrations. The contrast between places with high and medium traffic density and the two control areas was clearly manifested in PAH and Pb levels in the ambient air. Our data on concentrations of Pb, Cd, Zn, and BaP in the ambient air fit within the same range as the measurements obtained by the National Air Quality Monitoring Network. However, at two locations PM₁₀ values were higher than TSP values, whereas normally the PM₁₀ fraction is part of the TSP fraction. However, air particulate samples were taken on 2 different days and factors such as temperature, humidity, wind speed, and hours of sunshine might have an effect. Because of the limited number of measurements of particulate-bound pollutants in this study, no statistical tests were applied to these data.

Pb concentrations in particulate air samples were clearly reflected in Pb content of blood and organ tissue. Blood Pb levels of pigeons originating from the high and medium traffic density area in Amsterdam in this study were 235 ± 182 and 73 ± 19 ng/ml, respectively. These data fit within the same range as found in Japan (9) and London (12). The mean Pb levels in blood of pigeons derived from central Tokyo and suburban Tokyo were, respectively, 330 ± 204 and 54 ± 23 ng/ml (9), whereas in London blood Pb levels varied from 162 ± 55 to 1011 ± 132 ng/ml (12). Comparable values were obtained for Pb content in kidney. In the Amsterdam-high and -medium traffic density location, values of 2.4 ± 1.3 and 0.5 ± 0.2 μg/g wet weight, respectively, were measured in kidney tissue; in central and suburban Tokyo, mean Pb concentrations of 2.8 ± 2.2 and 0.8 ± 1.1 μg/g were found (9), and in London, average Pb concentrations varied from 64 ± 9 μg/g in central London to 2.0 ± 0.5 μg/g in the outer urban location of London (12). Mean Pb concentrations in blood and kidney in the two light-traffic density areas, Maastricht and Assen, were significantly lower; these values were within the same range as found in pigeons from a rural location in England (12). The high correlation between blood Pb concentrations and Pb content in the different organs indicates that actual exposure reflects chronic exposure reliably. Sources of Pb exposure include gasoline, atmospheric dust, street dust, paint, and natural sources.

The highest percentage of Pb from gasoline, namely 61.5%, was found in the high-traffic density location in Amsterdam followed by the medium-traffic density location in Amsterdam with 57.4%. Pigeons in Assen and Maastricht had, respectively, 56.2 and 48.9% Pb derived from gasoline in their blood. This is in agreement with studies on human blood. Colombo et al. (45) have shown in the Italian Turin area that more than 25% of Pb in human blood may be due to exposure to traffic exhaust. In children living in inner-city London, 30–40% of the Pb was derived from gasoline (33). We have recently analyzed blood of schoolchildren in The Netherlands (J.A. Hoogewerff, et al.).

Figure 4. Correlation between ratio of 7-hydro-8-oxo-2'-deoxyguanosine/2'-deoxyguanosine (8-oxoG/ddG) (10⁻⁶) and Pb concentrations in liver (ng/g, wet weight) in wild city pigeons from Amsterdam-high, Amsterdam-medium, Assen, and Maastricht. Regression analysis; p<0.05; r = 0.539.

Figure 5. Representative DNA adduct profiles of ³²P-postlabeled DNA digests from (A) liver, (B) kidney, and (C) lung from pigeons caught at the high-traffic density site in Amsterdam. (D) +/anti-benzo[a]pyrene diol epoxide (BPDE)-DNA standard (level of modification 1.10⁻⁶). The origin (bottom left-hand corner of the chromatograms) was excised after chromatography. Adduct profiles on the chromatograms were visualized and quantitated by using phosphor image analysis (Molecular Image; Model GS-250, Molecular Dynamics, Sunnyvale, CA). For comparison, panel D shows the adduct resulting from ³²P-labeling of calf thymus DNA reacted in vitro with BPDE, which is the main reactive metabolite of benzo[a]pyrene. In each experiment [³²P]BPDE-DNA standards with known modification levels (1 adduct per 10⁻⁷–10⁻⁹ nucleotides) were run parallel for quantitation purposes.
Moreover, the composition in these children is similar to the range for pigeons, which indicates that the amount of Pb from gasoline in these children fits within the same range as found in pigeons. Moreover, the composition of Pb in The Netherlands has a $^{206}$Pb/$^{207}$Pb ratio between 1.14 and 1.17.

In the Japanese study (9), aerosol Pb concentrations were measured in outside air. These values varied between 0.35 to 1.27 µg/m$^3$ in central Tokyo to 0.16 µg/m$^3$ in suburban Tokyo. The authors concluded that the respiratory route predominantly explained the differences in lead concentrations between the two groups of pigeons. However, only total Pb concentrations were measured, and therefore no discrimination could be made between the different sources of Pb.

In our study Pb concentrations in outside air varied from 37.1 ng/m$^3$ in the high-traffic density location of Amsterdam to on average 25.3 ng/m$^3$ Pb in the two control areas. These values are far below the concentrations found in Japan, which may be partly due to the introduction of lead-free gasoline in recent years in The Netherlands. In 1986 the maximum allowed lead content in gasoline decreased from 0.4 g/l to 0.15 g/l in The Netherlands. Due to the introduction of this lead-free gasoline, a sharp decline in lead air concentrations was observed. In 1985, annual average lead concentration in outside air was about 200 ng/m$^3$, while in 1993 average values of 40 ng/m$^3$ were measured (44).

Cd levels in liver and kidney fit well within the range reported for the pigeons obtained in London (12) and for other bird species (46). Cd contents in kidney and liver were significantly higher in Amsterdam as compared to the two control areas. The Cd levels in blood and lung were, however, comparable in the different regions and did not correlate with tissue levels. The differences in Cd concentrations in the ambient air corresponded to blood Cd concentrations in pigeons but not to tissue content. The Cd concentrations in whole blood reflect actual exposure, indicating previous higher exposure due to industrial activities and/or other sources or a higher rate of accumulation in pigeons from Amsterdam.

For Zn opposite results were found. Blood Zn values for pigeons from Amsterdam were significantly higher compared to the two other areas; this was, however, not reflected in Zn tissue levels. A negative correlation between blood Pb levels and body burden of Zn has been reported previously (47). Moreover, similar findings were observed in the pigeons from London (12). It is possible that competitive inhibition between these metal results in a decreased uptake of Zn as is reflected in actual tissue levels, but the lack of difference in Zn tissue levels between the four different locations was in agreement with Zn concentrations in the ambient air.

Of particular interest are the similarities in distribution pattern in tissues of the metals between birds and humans. Pb predominantly accumulated in kidney, followed by liver and lung. Cd levels were highest in kidney, followed by the liver, whereas much lower levels were found in lung tissue. For Zn comparable contents were found in liver and kidney, while lung Zn concentrations were two times lower.

It has been demonstrated that most metal ions do not form covalent adducts with DNA (48). This indicates that metal mutagenesis may proceed via the generation of activated intermediates such as reactive oxygen species, which has, in fact, been proven in several studies (48-53). Oxidative DNA damage in liver DNA of pigeons from the Amsterdam high-traffic density location was significantly enhanced compared to pigeons from the three other areas. Moreover, oxidative DNA damage showed a positive correlation with Pb levels in the liver, which indicates that the carcinogenic potential of Pb might be ascribed to oxygen radical formation. In lung tissue oxidative DNA damage appeared to be negatively correlated with Zn tissue levels, suggesting a protective role for Zn in the induction process of oxidative DNA damage. This is in agreement with previous reports in which the protective role of Zn on Pb toxicity has been described (54).

Pigeons are exposed to a mixture of environmental mutagenic agents. It is therefore extremely difficult to establish a causal relationship between DNA adducts and one of the components encountered in the environment because these agents may interfere with each other to produce synergistic or antagonistic effects. Toxic and carcinogenic effects of these metals can be modified by concurrent exposure to other metals.

Carcinogenic PAHs are efficiently metabolized into more polar, excretatable metabolites and may therefore, as such, not be easily detected in organisms. For biomonitoring studies, measurements of specific DNA adducts may provide more suitable biological markers of environmental exposure to carcinogenic compounds than measurements of the parent compound itself. It has been shown that in eels living in PAH-polluted water, no high molecular weight PAHs (e.g., PAHs containing four rings or more) could be found, but high levels of liver DNA adducts were detected (55).

In the present study, relatively high DNA adduct levels in the liver, kidney, and to a lesser extent in the lung, of pigeons were observed. We found in the lung, by $^{32}$P-postlabeling, a diffuse diagonal zone of aromatic DNA adducts, indicating a broad spectrum of adducts (Fig. 5B). Similar DRZs have been observed in several species, including humans, following exposure to complex mixtures. For example, tobacco-smoking-associated DRZs have been found in a variety of human tissues including lung, heart, and placenta (15,16). Furthermore, in rodents DRZs have been shown after exposure to cigarette smoke condensate (56), coal tar, creosote, or bitumen (57), and particulate extracts from coke oven, coal soot, and diesel exhaust (58) in skin, lung, heart, and liver. In line with our results, other $^{32}$P-postlabeling DNA adduct analyses have shown similar DRZs in fish obtained from polluted sites (17,19,59).

In the DNA adduct profiles of liver and kidney, an increased density within the DRZ could be observed, which could possibly present major adducts (Fig. 5A,B); the identity of these adducts is unknown. Separated adducts as observed in liver and kidney are probably related to more specific exposures, and other routes of exposure such as contaminated food, might be involved. Another striking observation was that the DRZ adduct levels are higher in kidney and liver than in the lung. A possible explanation may be that in liver, and to a lesser extent in kidney, cytochrome P450, responsible for the formation of reactive metabolites, is more abundant than in lung tissue. We also suggest that other parameters as cell proliferation and DNA repair might be involved.

No significant differences in aromatic DNA adduct levels were observed in tissues from pigeons caught at polluted sites compared with pigeons from reference sites. PAH concentrations in the outside air varied from 2.94 ng/m$^3$ in the high-traffic density location in Amsterdam to 0.59 and 1.33 ng/m$^3$ in the control areas Maastricht and Assen, respectively. This was not reflected in tissue DNA adduct levels. Therefore, this parameter does not seem to yield a relevant biomarker for exposure to mutagenic and carcinogenic PAHs as a result of traffic exhaust.

From these results, it appears that pigeons can be used for biomonitoring of exposure to heavy metals. The most striking findings are the regional differences of Pb and Cd pollution as reflected in body load of the pigeon. Moreover, the contribution of Pb from gasoline can be deter-
mended on the basis of analysis of the Pb isotopic composition in blood, which also resulted in marked regional differences, as has been shown in this study.

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