10 mg PFTeDA/kg/day in the main group on days 5 and 10 of gestation and on day 4 of lactation (data not shown).

At the end of the administration period, the hindlimb grip strength of male rats decreased in a dose-dependent manner, and a significant difference from the control was found in the 3 and 10 mg/kg/day groups (Fig. 2). No significant changes were observed in grip strength in males of the recovery group or in females. Furthermore, no significant differences were observed in urinalysis parameters between the PFTeDA-treated and control groups, either at the end of the administration period or at the end of the recovery period (data not shown).

In the main group, the only significant effect observed on hematology was a shortening in APTT in males given 10 mg PFTeDA/kg/day (Table 1). Blood biochemical examinations showed significant decreases in total protein in males and the β-globulin fraction in both sexes at 10 mg/kg/day (Table 1). Significant increases were also observed in ALP and BUN in males and Cl in females in the 10 mg/kg/day group. Absolute and relative liver weights were significantly increased at 3 and 10 mg/kg/day in males (Table 1). A significant increase in the relative liver weight was also found in females at 10 mg/kg/day. In males, the absolute weight of the pituitary gland was significantly decreased at 3 and 10 mg/kg/day and the relative weight was also significantly decreased at 3 mg/kg/day. The absolute weight of the seminal vesicle was significantly decreased at all doses.

Histopathologically, centrilobular hepatocyte hypertrophy was observed in males at 3 and 10 mg/kg/day and in females at 10 mg/kg/day (Table 2). Microgranulomas were noted in the liver of both sexes in all groups containing the control; however, the extent of these was significantly higher in females given 10 mg PFTeDA/kg/day. Focal necrosis was detected in the liver of one female given 10 mg PFTeDA/kg/day. Follicular cell hypertrophy was observed in the thyroids of males at 3 and 10 mg/kg/day. In females, the incidences of decreases in extramedullary hematopoiesis in the spleen and cortex atrophy in the thymus were significantly increased at 10 mg/kg/day. No treatment-related changes were detected in histopathology in other organs, including the pituitary gland and seminal vesicle.

In the recovery group, the hemoglobin concentration and hematocrit value were significantly decreased, and PT was significantly shortened in females in the 10 mg/kg/day group (Table 1). Significant increases were also observed in ALP and IP and decreases in triglyceride levels in males, as well as a significant decrease in total cholesterol and increase in BUN in females in the 10 mg/kg/day group. In this group, the absolute and/or relative liver weights were significantly increased, and histopathologically, centrilobular hypertrophy of hepatocytes, diffuse hypertrophy of hepatocytes or diffuse fatty change was found in the liver (Table 2). Hypertrophy of follicular cells was observed in the thyroids of two males in the 10 mg/kg/day group.

*Reproductive/developmental toxicity*

Reproductive/developmental results are summarized in Table 3. No significant changes were found in reproduc-

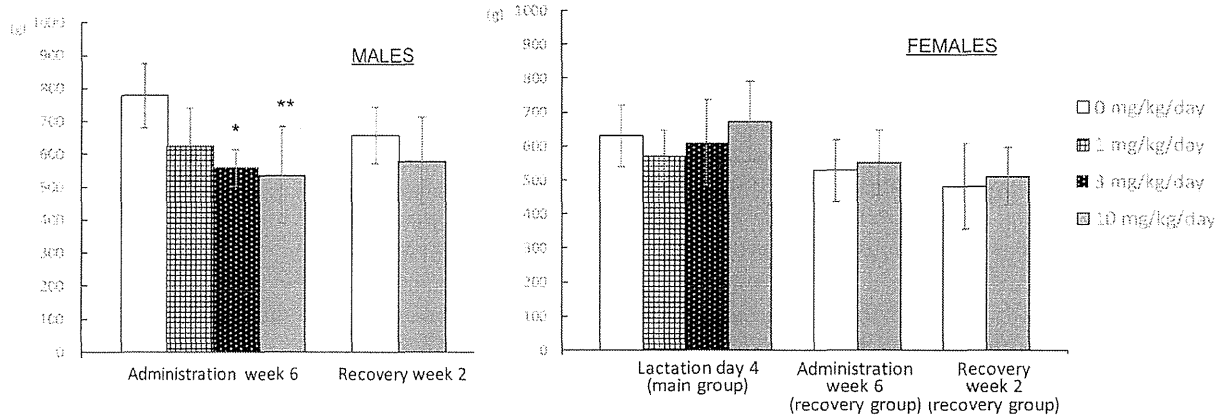


**Fig. 2.** The hindlimb grip strength of male and female rats in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for PFTeDA. *: Significantly different from the control, $P \leq 0.05$. **: Significantly different from the control, $P \leq 0.01$.

**Table 1.** Significant changes in hematological and blood biochemical parameters and organ weights in rats given PFTeDA.

| Dose (mg/kg/day) | At the end of the administration period (Main group) | | | | At the end of the recovery period (Recovery group) | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 10 | 0 | 10 |
| MALES | | | | | | |
| *Hematology* | | | | | | |
| Hemoglobin (g/dL) | 15.78 ± 0.61 | 16.00 ± 0.85 | 15.90 ± 0.55 | 15.98 ± 0.77 | 16.42 ± 0.38 | 15.88 ± 0.61 |
| Hematocrit (%) | 44.86 ± 2.02 | 45.26 ± 2.10 | 44.80 ± 1.81 | 45.16 ± 2.38 | 46.16 ± 1.04 | 45.50 ± 1.99 |
| PT (sec) | 21.56 ± 6.33 | 22.46 ± 0.78 | 22.74 ± 2.34 | 21.78 ± 4.27 | 20.10 ± 3.33 | 21.14 ± 1.62 |
| APTT (sec) | 27.42 ± 3.61 | 28.18 ± 1.36 | 26.42 ± 1.97 | 23.62 ± 1.28* | 25.78 ± 2.34 | 24.68 ± 3.86 |
| *Blood biochemistry* | | | | | | |
| Total protein (g/dL) | 5.74 ± 0.19 | 5.66 ± 0.21 | 5.78 ± 0.30 | 5.26 ± 0.11** | 5.80 ± 0.12 | 5.52 ± 0.34 |
| β-Globulin fraction of protein (%) | 17.12 ± 0.91 | 16.14 ± 0.80 | 16.24 ± 0.57 | 15.68 ± 0.99* | 16.40 ± 0.72 | 16.36 ± 0.51 |
| ALP (IU/L) | 363.2 ± 81.5 | 352.6 ± 113.1 | 355.0 ± 51.8 | 520.8 ± 75.6* | 334.2 ± 51.7 | 470.4 ± 67.3** |
| Triglyceride (mg/dL) | 36.0 ± 9.4 | 50.4 ± 22.1 | 46.4 ± 17.1 | 21.8 ± 6.3 | 59.2 ± 14.2 | 31.8 ± 8.9** |
| Total cholesterol (mg/dL) | 60.0 ± 9.9 | 53.8 ± 6.0 | 44.0 ± 11.2 | 50.8 ± 14.7 | 63.8 ± 18.9 | 50.6 ± 10.7 |
| BUN (mg/dL) | 14.26 ± 1.43 | 14.02 ± 1.47 | 16.00 ± 1.71 | 19.88 ± 1.99** | 14.82 ± 1.04 | 16.20 ± 2.11 |
| Cl (mEq/L) | 106.8 ± 1.3 | 107.4 ± 1.8 | 107.0 ± 0.7 | 108.4 ± 2.3 | 105.4 ± 1.1 | 106.6 ± 1.1 |
| IP (mg/dL) | 6.36 ± 0.47 | 6.04 ± 0.50 | 6.42 ± 0.60 | 6.98 ± 0.44 | 6.20 ± 0.33 | 6.68 ± 0.29* |
| *Organ weight* | | | | | | |
| Liver (g) | 11.95 ± 1.53 | 12.09 ± 0.73 | 14.52 ± 1.82* | 15.21 ± 0.53** | 13.09 ± 0.95 | 16.41 ± 0.48** |
| (%) | 2.41 ± 0.11 | 2.49 ± 0.12 | 2.87 ± 0.23** | 3.25 ± 0.07** | 2.43 ± 0.15 | 3.18 ± 0.16** |
| Pituitary gland (mg) | 13.20 ± 0.74 | 13.06 ± 1.17 | 11.18 ± 0.87* | 11.48 ± 1.07* | 13.10 ± 2.46 | 13.70 ± 1.04 |
| ($10^{-3}$%) | 2.69 ± 0.28 | 2.69 ± 0.26 | 2.22 ± 0.21* | 2.46 ± 0.28 | 2.43 ± 0.44 | 2.65 ± 0.21 |
| Seminal vesicle (g) | 2.53 ± 0.51 | 2.00 ± 0.19* | 2.01 ± 0.29* | 1.91 ± 0.15* | 2.18 ± 0.12 | 2.14 ± 0.42 |
| (%) | 0.512 ± 0.102 | 0.412 ± 0.035 | 0.402 ± 0.077 | 0.408 ± 0.023 | 0.402 ± 0.029 | 0.414 ± 0.074 |
| FEMALES | | | | | | |
| *Hematology* | | | | | | |
| Hemoglobin (g/dL) | 15.14 ± 0.40 | 14.68 ± 0.70 | 15.02 ± 0.87 | 15.50 ± 0.60 | 15.68 ± 0.62 | 14.46 ± 0.68* |
| Hematocrit (%) | 43.94 ± 2.17 | 43.24 ± 2.58 | 44.26 ± 2.83 | 44.94 ± 1.48 | 44.34 ± 1.67 | 40.90 ± 1.95* |
| PT (sec) | 18.78 ± 0.93 | 17.60 ± 1.04 | 17.76 ± 0.47 | 17.52 ± 1.32 | 17.26 ± 0.50 | 16.12 ± 0.87* |
| APTT (sec) | 19.00 ± 0.46 | 19.54 ± 0.52 | 19.88 ± 0.60 | 19.44 ± 0.75 | 18.28 ± 1.30 | 19.48 ± 1.03 |
| *Blood biochemistry* | | | | | | |
| Total protein (g/dL) | 6.30 ± 0.22 | 6.52 ± 0.25 | 6.40 ± 0.23 | 6.12 ± 0.44 | 6.46 ± 0.32 | 6.30 ± 0.28 |
| β-Globulin fraction of protein (%) | 18.32 ± 0.54 | 17.32 ± 1.15 | 17.56 ± 0.98 | 15.90 ± 0.92** | 14.86 ± 0.98 | 14.54 ± 0.48 |
| ALP (IU/L) | 196.8 ± 54.0 | 174.0 ± 33.7 | 177.8 ± 51.3 | 236.0 ± 32.1 | 177.6 ± 51.8 | 234.2 ± 72.9 |
| Triglyceride (mg/dL) | 49.6 ± 29.2 | 43.0 ± 10.9 | 47.6 ± 13.9 | 29.2 ± 18.0 | 15.8 ± 7.7 | 18.8 ± 16.5 |
| Total cholesterol (mg/dL) | 68.8 ± 12.2 | 63.0 ± 14.5 | 57.2 ± 10.9 | 51.2 ± 15.4 | 75.8 ± 11.6 | 56.6 ± 10.6* |
| BUN (mg/dL) | 25.58 ± 2.73 | 24.08 ± 2.27 | 23.80 ± 3.17 | 30.44 ± 4.18 | 15.00 ± 1.60 | 20.24 ± 4.60* |
| Cl (mEq/L) | 102.6 ± 2.3 | 103.8 ± 0.8 | 105.0 ± 1.6 | 105.8 ± 0.8* | 108.4 ± 2.2 | 107.4 ± 1.1 |
| IP (mg/dL) | 9.08 ± 0.91 | 8.86 ± 0.79 | 8.20 ± 0.45 | 8.30 ± 0.60 | 4.70 ± 0.85 | 5.64 ± 0.61 |
| *Organ weight* | | | | | | |
| Liver (g) | 10.14 ± 0.75 | 10.96 ± 1.13 | 10.17 ± 0.19 | 10.44 ± 0.89 | 7.18 ± 0.69 | 7.90 ± 1.07 |
| (%) | 3.33 ± 0.17 | 3.49 ± 0.21 | 3.42 ± 0.16 | 3.70 ± 0.29* | 2.40 ± 0.27 | 2.83 ± 0.27* |
| Pituitary gland (mg) | 16.26 ± 2.43 | 17.44 ± 1.80 | 16.54 ± 0.93 | 15.82 ± 2.99 | 16.32 ± 2.29 | 18.06 ± 3.75 |
| ($10^{-3}$%) | 5.37 ± 0.97 | 5.58 ± 0.62 | 5.56 ± 0.24 | 5.67 ± 1.37 | 5.44 ± 0.63 | 6.42 ± 1.04 |

Data are shown as the mean ± S.D.

*: Significantly different from the control group at $P \leq 0.05$.

**: Significantly different from the control group at $P \leq 0.01$.

**Table 2.** Histopathological findings in the combined repeated dose toxicity study with reproduction/developmental toxicity screening test for PFTeDA in rats.

| Dose (mg/kg/day) | | At the end of the administration period (Main group) | | | | At the end of the recovery period (Recovery group) | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 3 | 10 | 0 | 10 |
| MALES | | | | | | | |
| Number of examined animals | | 7 | 12 | 12 | 7 | 5 | 5 |
| Liver | | | | | | | |
| - Centrilobular hypertrophy of hepatocytes | + | 0 | 0 | 6 ]* | 0 ]** | 0 | 2 ]** |
| | ++ | 0 | 0 | 2 ]* | 7 ]** | 0 | 3 ]** |
| - Microgranuloma | + | 4 | 10 | 6 | 1 | 3 | 4 |
| - Diffuse fatty change | + | 0 | 0 | 0 | 0 | 0 | 2 |
| Thyroid | | | | | | | |
| - Hypertrophy of follicular cells | + | 0 | 0 | 4 | 4 | 0 | 2 |
| FEMALES | | | | | | | |
| Number of examined animals | | 12 | 12 | 12 | 12 | 5 | 5 |
| Liver | | | | | | | |
| - Centrilobular hypertrophy of hepatocytes | + | 0 | 0 | 0 | 9** | 0 | 2 |
| - Diffuse hypertrophy of hepatocytes | + | 0 | 0 | 0 | 0 ]** | 0 | 2 |
| - Microgranuloma | + | 6 | 9 | 8 | 3 ]** | 4 | 1 |
| | ++ | 0 | 0 | 0 | 7 | 0 | 3 |
| - Focal necrosis | + | 0 | 0 | 0 | 1 | 0 | 0 |
| Spleen | | | | | | | |
| - Decrease in extramedullary hematopoiesis | + | 2 | 0 | 2 | 8* | 0 | 0 |
| Thymus | | | | | | | |
| - Cortex atrophy | + | 1 | 2 | 1 | 8** | 0 | 0 |

Values represent the number of animals with findings.
+: Slight change, ++: moderate change
*: Significantly different from the control group at $P \leq 0.05$.
**: Significantly different from the control group at $P \leq 0.01$.
Brackets in the data columns mean that statistical analysis was performed for a total number of animals with findings in consideration of grades.

tive parameters, including estrous cyclicity, the copulation index, fertility index, gestation index or gestation length. No significant differences were observed in the number of corpora lutea, implantation sites, delivered pups, or live pups on PNDs 0 and 4, or in the sex ratio of live pups between the PFTeDA-treated and control groups. In the 10 mg/kg/day group, the body weights of male and female pups were significantly lower on PNDs 1 and 4. There were no abnormalities in the general appearance or necropsy findings of neonates.

### Perfluorohexadecanoic acid (PFHxDA: C16)

*Repeated dose toxicity*

No treatment-related clinical signs of toxicity were observed throughout the study. The body weights of males in the 100 mg/kg/day group were significantly lower than those of the control on days 35 and 42 of the administration period (Fig. 3). Such effects on body weight were not detected in the females. Food consumption was significantly reduced on day 14 of the recovery period in males given 100 mg PFHxDA/kg/day, on days 5-14 of the gestation period, and on day 4 of the lactation period in females given 100 mg PFHxDA/kg/day in the main group (data not shown).

A functional observation at the end of the administration period revealed no significant changes in any of the PFHxDA-treated groups, but a significant decrease in hindlimb grip strength at the end of recovery period in both sexes given 100 mg PFHxDA/kg/day (Fig. 4). No significant difference was seen in any urinalysis parameters between the control and PFHxDA-treated groups either at the end of the administration period or at the end of the recovery period (data not shown).

At the end of the administration period, no significant differences were observed in any hematological parameters between the control and PFHxDA-treated groups

**Table 3.** Reproductive/developmental findings in the combined repeated dose toxicity study with the reproduction/developmental screening test for PFTeDA in rats.

| Dose (mg/kg/day) | | 0 | 1 | 3 | 10 |
|---|---|---|---|---|---|
| Incidence of females with normal estrous cycle[a] (%) | | 91.7 | 91.7 | 91.7 | 100 |
| Estrous cycle length[a, b] (days) | | 4.06 ± 0.21 | 4.00 ± 0.00 | 3.98 ± 0.17 | 4.06 ± 0.20 |
| Number of cohabited pairs | | 12 | 12 | 12 | 12 |
| Copulation index (%) | Males | 91.7 | 91.7 | 100 | 100 |
| | Females | 100 | 100 | 100 | 100 |
| Fertility index (%) | | 100 | 100 | 91.7 | 100 |
| Gestation index (%) | | 100 | 100 | 100 | 100 |
| Gestation length[b] (days) | | 22.3 ± 0.7 | 22.3 ± 0.5 | 22.2 ± 0.4 | 22.0 ± 0.0 |
| Number of pregnant females | | 12 | 12 | 11 | 12 |
| Number of corpora lutea[b] | | 16.7 ± 1.9 | 16.4 ± 1.8 | 16.1 ± 1.6 | 17.0 ± 2.2 |
| Number of implantation sites[b] | | 16.0 ± 1.7 | 16.2 ± 1.6 | 15.9 ± 1.8 | 16.4 ± 2.0 |
| Number of pups delivered[b] | | 14.5 ± 3.8 | 15.3 ± 2.0 | 15.3 ± 2.1 | 15.8 ± 1.8 |
| Sex ratio of pups (male pups / all pups)[b] | | 0.470 ± 0.113 | 0.532 ± 0.101 | 0.481 ± 0.132 | 0.547 ± 0.116 |
| Number of live pups[b] | on PND 0 | 14.5 ± 3.8 | 15.3 ± 2.0 | 15.2 ± 2.0 | 15.8 ± 1.8 |
| | on PND 4 | 14.1 ± 3.6 | 15.0 ± 1.9 | 15.1 ± 1.8 | 15.2 ± 1.3 |
| Body weight of male pups[b] (g) | | | | | |
| | on PND 0 | 6.58 ± 0.93 | 6.62 ± 0.76 | 6.43 ± 0.41 | 6.01 ± 0.34 |
| | on PND 1 | 7.32 ± 1.14 | 7.19 ± 0.89 | 6.97 ± 0.52 | 6.31 ± 0.46** |
| | on PND 4 | 10.66 ± 2.03 | 10.53 ± 1.31 | 9.93 ± 0.76 | 8.77 ± 0.85** |
| Body weight of female pups[b] (g) | | | | | |
| | on PND 0 | 6.29 ± 0.81 | 6.28 ± 0.68 | 6.05 ± 0.34 | 5.78 ± 0.36 |
| | on PND 1 | 6.99 ± 1.03 | 6.83 ± 0.78 | 6.53 ± 0.49 | 6.05 ± 0.45** |
| | on PND 4 | 10.18 ± 1.72 | 9.98 ± 1.21 | 9.35 ± 0.68 | 8.41 ± 0.85** |

a: Data of the main group are shown. No significant changes in estrous cycle normality were found in the recovery group, either.
b: Data are shown as the mean ± S.D.
*: Significantly different from the control group at $P \leq 0.05$.
**: Significantly different from the control group at $P \leq 0.01$.

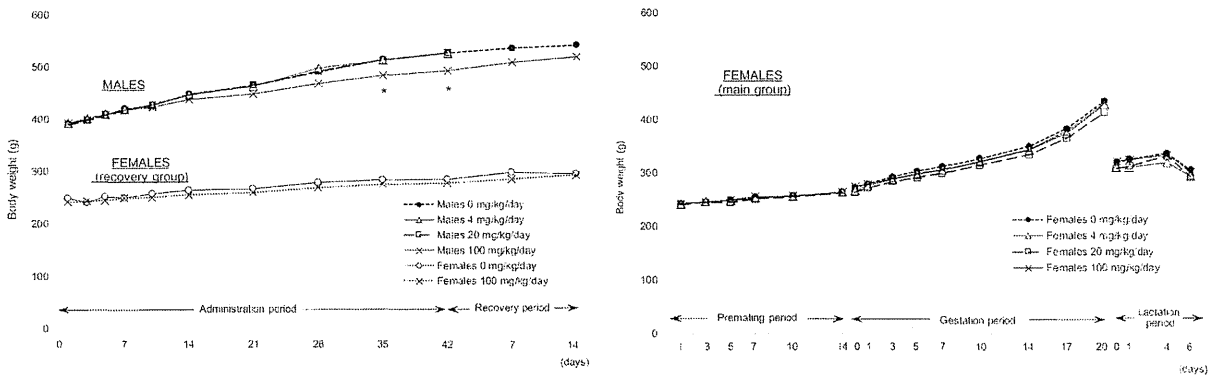


**Fig. 3.** Body weight changes in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for PFHxDA in rats. *: Significantly different from the control, $P \leq 0.05$.

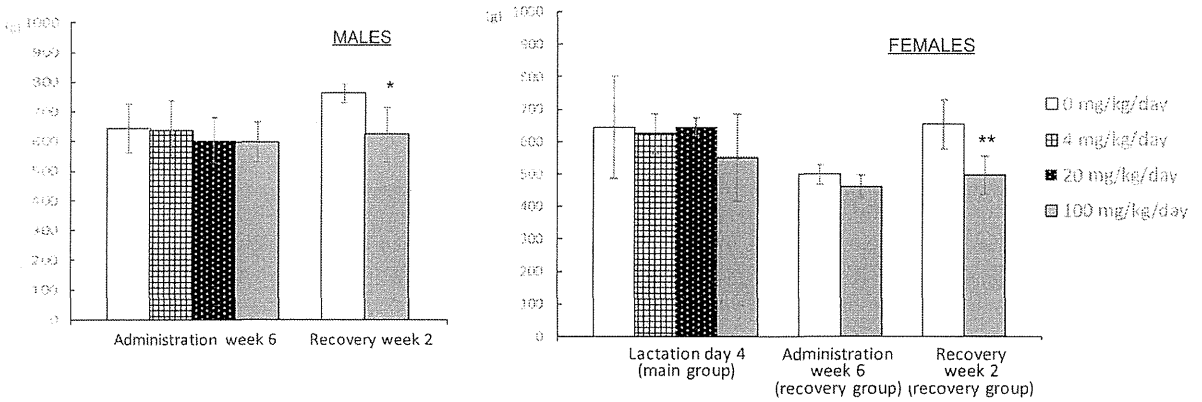


**Fig. 4.** The hindlimb grip strength of male and female rats in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for PFHxDA. *: Significantly different from the control, $P \leq 0.05$. **: Significantly different from the control, $P \leq 0.01$.

(data not shown). Serum Cl levels were significantly increased at 100 mg/kg/day in males and at 20 and 100 mg/kg/day in females (Table 4). A significant decrease in serum total bilirubin levels and significant increases in BUN and serum Na levels were also detected in females given 100 mg PFHxDA/kg/day. In males, the absolute and relative liver weights were significantly increased in the 100 mg/kg/day group (Table 4). The relative thyroid weight was significantly increased at 20 and 100 mg/kg/day, with a significant increase also being observed in the absolute weight at 20 mg/kg/day in males. The analysis of serum thyroid-related hormones revealed significantly decreased $T_3$ in females in all PFHxDA-treated groups. The histopathological examination revealed the centrilobular hypertrophy of hepatocytes in males at 20 mg/kg/day and in both sexes at 100 mg/kg/day (Table 5). Centrilobular fatty changes were also observed in males at 20 and 100 mg/kg/day. No treatment-related histopathological changes were detected in other organs including the thyroid.

A significant decrease was noted in serum total bilirubin levels in both sexes as well as a significant increase in serum Cl level in females in the 100 mg/kg/day group after the 14-day recovery period (Table 4). Serum $T_4$ levels were significantly decreased in males in the 100 mg/kg/day group. Absolute and relative liver weights in males still remained higher, and in addition, significant decreases were found in absolute and relative adrenal weights in the 100 mg/kg/day group. Histopathologically, the centrilobular hypertrophy of hepatocytes was observed in both sexes as well as centrilobular fatty changes in one male in the 100 mg/kg/day group (Table 5).

*Reproductive/developmental toxicity*

PFHxDA did not significantly affect any reproductive/developmental parameters (Table 6). Although the body weights of male and female pups on PND 4 were slightly lower in the 100 mg/kg/day group, no significant difference was observed from those in the control group. There were no abnormalities in the general appearance or necropsy findings of neonates.

## DISCUSSION

The present study was performed to obtain initial information on the repeated dose and reproductive/developmental toxicity of PFTeDA (C14) and PFHxDA (C16). The results obtained demonstrated that the main toxic target of these compounds was the liver, which was similar to PFUnA (C11), PFDoA (C12), and PFOcDA (C18), which we had examined previously (Hirata-Koizumi *et al.*, 2012; Kato *et al.*, in press; Takahashi *et al.*, 2014).

The hepatic effects of PFCAs in rodents have been attributed, at least partly, to the peroxisome proliferator-activated receptor alpha (PPARα) (Lau *et al.*, 2007; Wolf *et al.*, 2012). PPARα is a nuclear receptor that plays an important role in regulating fatty acid metabolism in tissues such as the liver, kidney, heart, and intestinal mucosa (Corton *et al.*, 2000). In the present study, the blood biochemical examination did not reveal any clear effects on lipid metabolism; however, PFTeDA (C14) and PFHxDA (C16) decreased serum total cholesterol in the 14-day dose finding study performed at higher doses. Although the PPARα agonist activities of PFTeDA and PFHxDA are unknown, PFDoA (C12), which is very similar in

**Table 4.** Significant changes in blood biochemical parameters, serum thyroid-related hormone levels and organ weights in rats given PFHxDA.

| Dose (mg/kg/day) | At the end of the administration period (Main group) | | | | At the end of the recovery period (Recovery group) | |
|---|---|---|---|---|---|---|
| | 0 | 4 | 20 | 100 | 0 | 100 |
| MALES | | | | | | |
| *Blood biochemistry* | | | | | | |
| Total bilirubin (mg/dL) | 0.062 ± 0.008 | 0.056 ± 0.011 | 0.058 ± 0.015 | 0.064 ± 0.011 | 0.064 ± 0.013 | 0.044 ± 0.005* |
| BUN (mg/dL) | 14.54 ± 0.88 | 14.52 ± 1.54 | 14.98 ± 1.60 | 17.52 ± 2.81 | 16.16 ± 1.18 | 15.12 ± 1.31 |
| Na (mEq/L) | 144.2 ± 1.6 | 144.4 ± 1.7 | 144.4 ± 1.1 | 145.4 ± 0.9 | 144.2 ± 1.5 | 144.4 ± 0.5 |
| Cl (mEq/L) | 106.8 ± 1.3 | 108.2 ± 2.3 | 107.4 ± 0.9 | 109.6 ± 1.5* | 106.2 ± 1.1 | 108.0 ± 1.4 |
| *Hormonalysis* | | | | | | |
| $T_3$ (ng/mL) | 0.450 ± 0.070 | 0.466 ± 0.076 | 0.390 ± 0.060 | 0.436 ± 0.119 | 0.474 ± 0.123 | 0.452 ± 0.061 |
| $T_4$ (ng/mL) | 69.71 ± 14.91 | 74.73 ± 10.93 | 80.05 ± 8.65 | 71.38 ± 3.83 | 117.50 ± 15.00 | 89.25 ± 11.87* |
| TSH (ng/mL) | 3.732 ± 1.491 | 6.586 ± 2.712 | 7.064 ± 5.351 | 9.682 ± 6.029 | 13.314 ± 5.530 | 13.564 ± 3.229 |
| *Organ weight* | | | | | | |
| Liver (g) | 12.15 ± 1.27 | 11.81 ± 0.55 | 12.12 ± 0.85 | 14.50 ± 0.61** | 12.38 ± 1.40 | 14.62 ± 1.35* |
| (%) | 2.50 ± 0.04 | 2.45 ± 0.10 | 2.49 ± 0.15 | 3.26 ± 0.07** | 2.40 ± 0.17 | 2.97 ± 0.33** |
| Thyroid (mg) | 18.94 ± 1.6 | 20.58 ± 1.53 | 24.26 ± 4.28* | 22.16 ± 3.26 | 21.90 ± 3.98 | 22.40 ± 4.10 |
| ($10^{-3}$%) | 3.94 ± 0.58 | 4.27 ± 0.34 | 4.98 ± 0.78* | 4.98 ± 0.67* | 4.26 ± 0.82 | 4.54 ± 0.84 |
| Adrenal (mg) | 70.0 ± 2.1 | 58.4 ± 11.9 | 62.4 ± 9.9 | 57.8 ± 8.4 | 70.4 ± 3.8 | 55.2 ± 6.1** |
| ($10^{-3}$%) | 14.52 ± 1.43 | 12.09 ± 2.41 | 12.91 ± 2.56 | 13.03 ± 2.03 | 13.69 ± 0.72 | 11.17 ± 1.14** |
| FEMALES | | | | | | |
| *Blood biochemistry* | | | | | | |
| Total bilirubin (mg/dL) | 0.080 ± 0.007 | 0.076 ± 0.005 | 0.072 ± 0. 013 | 0.060 ± 0.000** | 0.084 ± 0.015 | 0.054 ± 0.011** |
| BUN (mg/dL) | 25.78 ± 2.35 | 27.82 ± 2.05 | 28.22 ± 4.41 | 31.18 ± 1.55* | 16.66 ± 1.08 | 15.50 ± 1.09 |
| Na (mEq/L) | 140.8 ± 0.8 | 142.2 ± 0.8 | 142.6 ± 1.5 | 142.8 ± 1.3* | 144.6 ± 1.1 | 145.0 ± 0.7 |
| Cl (mEq/L) | 104.0 ± 0.7 | 105.0 ± 0.7 | 106.2 ± 1.3* | 106.8 ± 1.6** | 108.8 ± 0.8 | 109.8 ± 0.4* |
| *Hormonalysis* | | | | | | |
| $T_3$ (ng/mL) | 0.734 ± 0.023 | 0.606 ± 0.036** | 0.626 ± 0.068** | 0.532 ± 0.040** | 0.784 ± 0.143 | 0.684 ± 0.032 |
| $T_4$ (ng/mL) | 65.48 ± 9.30 | 65.56 ± 15.86 | 61.86 ± 7.57 | 66.36 ± 14.85 | 46.26 ± 16.70 | 58.48 ± 7.11 |
| TSH (ng/mL) | 4.478 ± 1.454 | 5.434 ± 5.130 | 4.408 ± 2.329 | 8.338 ± 4.661 | 3.758 ± 0.859 | 28.772 ± 54.988 |
| *Organ weight* | | | | | | |
| Liver (g) | 10.17 ± 0.48 | 9.70 ± 0.61 | 10.00 ± 0.81 | 10.53 ± 0.75 | 6.95 ± 0.37 | 7.48 ± 1.00 |
| (%) | 3.39 ± 0.12 | 3.27 ± 0.21 | 3.35 ± 0.15 | 3.55 ± 0.20 | 2.49 ± 0.11 | 2.71 ± 0.28 |
| Thyroid (mg) | 19.28 ± 3.06 | 15.78 ± 2.95 | 16.96 ± 3.42 | 18.04 ± 1.99 | 18.42 ± 1.97 | 16.88 ± 3.46 |
| ($10^{-3}$%) | 6.40 ± 0.78 | 5.30 ± 0.86 | 5.71 ± 1.27 | 6.07 ± 0.55 | 6.60 ± 0.79 | 6.09 ± 1.01 |
| Adrenal (mg) | 76.4 ± 5.8 | 79.6 ± 6.0 | 79.4 ± 7.9 | 75.4 ± 7.9 | 67.0 ± 3.5 | 74.2 ± 12.4 |
| ($10^{-3}$%) | 25.44 ± 1.68 | 26.80 ± 2.00 | 26.59 ± 2.12 | 25.43 ± 2.77 | 23.98 ± 1.11 | 26.95 ± 4.70 |

Data are shown as the mean ± S.D.
*: Significantly different from the control group at $P \leq 0.05$.
**: Significantly different from the control group at $P \leq 0.01$.

structure, was recently reported to activate mouse PPARα in transiently transfected COS-1 cells (Wolf *et al.*, 2012) and induce the mRNA levels of the important PPARα target genes, acyl CoA oxidase and CYP4A1, in the rat liver (Zhang *et al.*, 2008; Ding *et al.*, 2009). These findings indicated that PFTeDA and PFHxDA may activate PPARα, which may in turn affect the liver. Regarding the mechanism underlying the hepatotoxicity of PFCAs, many studies have examined PFOA (C8) and showed that PFOA could elicit changes in the liver not only via PPARα activation, but also through PPARα-independent mechanisms (Peters and Gonzalez, 2011). The involvement of other transcription factors such as the constitutive androstane receptor and pregnane X receptor has been implied. Further research is needed to clarify the mechanism involved in the hepatotoxicity of PFCAs including PFTeDA and PFHxDA.

PFTeDA (C14) induced follicular cell hypertrophy in

**Table 5.** Histopathological findings in the combined repeated dose toxicity study with reproduction/developmental toxicity screening test for PFHxDA in rats.

| Dose (mg/kg/day) | | At the end of the administration period (Main group) | | | | At the end of the recovery period (Recovery group) | |
|---|---|---|---|---|---|---|---|
| | | 0 | 4 | 20 | 100 | 0 | 100 |
| MALES | | | | | | | |
| Number of examined animals | | 7 | 12 | 12 | 7 | 5 | 5 |
| Liver | | | | | | | |
| - Centrilobular hypertrophy of hepatocytes | + | 0 | 0 | 5 | 0 ┐** | 0 | 5** |
| | ++ | 0 | 0 | 0 | 7 ┘ | 0 | 0 |
| - Centrilobular fatty change | + | 0 | 0 | 2 | 7** | 0 | 1 |
| FEMALES | | | | | | | |
| Number of examined animals | | 12 | 12 | 12 | 12 | 5 | 5 |
| Liver | | | | | | | |
| - Centrilobular hypertrophy of hepatocytes | + | 0 | 0 | 0 | 8** | 0 | 1 |

Values represent the number of animals with findings.
+: Slight change, ++: moderate change
**: Significantly different from the control group at $P \leq 0.01$.
Brackets in the data columns mean that statistical analysis was performed for a total number of animals with findings in consideration of grades.

**Table 6.** Reproductive/developmental findings in the combined repeated dose toxicity study with the reproduction/developmental screening test for PFHxDA in rats.

| Dose (mg/kg/day) | | 0 | 4 | 20 | 100 |
|---|---|---|---|---|---|
| Incidence of females with normal estrous cycle[a] (%) | | 100 | 100 | 100 | 100 |
| Estrous cycle length[a,b] (days) | | 4.11 ± 0.22 | 4.18 ± 0.32 | 4.03 ± 0.09 | 4.00 ± 0.00 |
| Number of cohabited pairs | | 12 | 12 | 12 | 12 |
| Couplation index (%) | Males | 100 | 100 | 100 | 100 |
| | Females | 100 | 100 | 100 | 100 |
| Fertility index (%) | | 91.7 | 100 | 100 | 100 |
| Gestation index (%) | | 100 | 100 | 100 | 100 |
| Gestation length[b] (days) | | 22.3 ± 0.5 | 22.3 ± 0.5 | 22.3 ± 0.5 | 22.2 ± 0.4 |
| Number of pregnant females | | 11 12 | 12 12 | | |
| Number of corpora lutea[b] | | 16.5 ± 1.1 | 17.0 ± 1.2 | 15.8 ± 1.9 | 16.1 ± 1.6 |
| Number of implantation sites[b] | | 16.1 ± 1.4 | 16.6 ± 1.2 | 15.3 ± 2.1 | 15.8 ± 1.6 |
| Number of pups delivered[b] | | 15.2 ± 1.7 | 16.0 ± 1.7 | 14.2 ± 2.2 | 14.6 ± 2.0 |
| Sex ratio of pups (male pups / all pups)[b] | | 0.505 ± 0.165 | 0.413 ± 0.158 | 0.429 ± 0.140 | 0.492 ± 0.183 |
| Number of live pups[b] | on PND 0 | 15.1 ± 1.7 | 15.8 ± 1.6 | 14.2 ± 2.2 | 14.5 ± 2.2 |
| | on PND 4 | 15.0 ± 1.7 | 13.1 ± 6.3 | 14.1 ± 2.3 | 13.7 ± 2.7 |
| Body weight of male pups[b] (g) | | | | | |
| | on PND 0 | 6.63 ± 0.58 | 6.67 ± 0.67 | 6.75 ± 0.69 | 6.45 ± 0.46 |
| | on PND 1 | 7.25 ± 0.56 | 7.12 ± 1.08 | 7.33 ± 0.75 | 6.97 ± 0.80 |
| | on PND 4 | 10.53 ± 0.85 | 10.63 ± 1.54 | 10.67 ± 1.14 | 9.93 ± 1.24 |
| Body weight of female pups[b] (g) | | | | | |
| | on PND 0 | 6.27 ± 0.51 | 6.22 ± 0.60 | 6.40 ± 0.66 | 6.08 ± 0.50 |
| | on PND 1 | 6.91 ± 0.51 | 6.58 ± 1.07 | 6.98 ± 0.82 | 6.59 ± 0.79 |
| | on PND 4 | 10.05 ± 0.79 | 9.97 ± 1.36 | 10.15 ± 1.25 | 9.43 ± 1.31 |

a: Data of the main group are shown. No significant changes in estrous cycle normality were found in the recovery group, either.
b: Data are shown as the mean ± S.D.

**Table 7.** Comparison of the NOAELs for the repeated dose and reproductive/developmental toxicity for long-chain PFCAs.

| Chemical name | Carbon number | NOAEL (mg/kg/day) | | Reference |
|---|---|---|---|---|
| | | Repeated dose toxicity | Reproductive /developmental toxicity | |
| PFUnA (perfluoroundacanoic acid) | 11 | 0.1 | 0.3 | Takahashi *et al.*, 2014 |
| PFDoA (perfluorododecanoic acid) | 12 | 0.1 | 0.5 | Kato *et al.*, in press |
| PFTeDA (perfluorotetradecanoic acid) | 14 | 1 | 3 | Current study |
| PFHxDA (perfluorohexadecanoic acid) | 16 | 4 | 100 | Current study |
| PFOcDA (perfluorooctadecanoic acid) | 18 | 40 | 200 | Hirata-Koizumi *et al.*, 2012 |

The NOAELs were established based on the results of in the combined repeated dose toxicity study with reproduction/developmental toxicity screening tests in rats

the thyroids of males. Although the serum levels of thyroid-related hormones were not analyzed in the present study for PFTeDA, it may be a compensatory response of the thyroid to a decrease in thyroid hormone levels because the structural analogue, perfluorodecanoic acid (PFDeA, C10), was previously reported to reduce serum $T_3$ and/or $T_4$ levels in rats (Gutshall *et al.*, 1988; Van Rafelghem *et al.*, 1987; Langley and Pilcher, 1985; Gutshall *et al.*, 1989). In the present study, PFHxDA (C16) did not affect the histopathology of thyroids, but increased the thyroid weight in males and decreased serum $T_3$ level in females. Although these effects of PFHxDA were not consistent between sexes and lacked clear dose-dependency, our results indicate that PFHxDA may slightly affect the thyroid system through a similar mechanism to PFTeDA (C14) and PFDeA (C10). The findings of mechanistic studies on PFDeA (C10) suggested that reduced serum thyroid hormone levels may result from (1) a displacement in the hormones from plasma protein binding sites, leading to an increase in tissue uptake and turnover (Gutshall *et al.*, 1989), and (2) the enhanced metabolism of thyroid hormones in the liver (Shelby and Klaassen, 2006). In our previous studies, we did not detect any effects of PFUnA (C11), PFDoA (C12), and PFOcDA (C18) on the histopathology or weight of the thyroids (Hirata-Koizumi *et al.*, 2012; Kato *et al.*, in press; Takahashi *et al.*, 2014). Serum hormone levels were not measured in these studies.

We previously reported that PFOcDA (C18) reduced forelimb grip strength in females (Hirata-Koizumi *et al.*, 2012). This effect was not observed at the end of the administration period, but appeared at the end of recovery period in both sexes in studies on PFUnA (C11) and PFDoA (C12) (Kato *et al.*, in press; Takahashi *et al.*, 2014). We considered that the reduction observed in grip strength may reflect the muscle weakness associated with a decrease in food consumption and/or body weight. In the present study, PFTeDA (C14) and PFHxDA (C16) reduced hindlimb grip strength, but not that of the forelimb. As with PFUnA (C11) and PFDoA (C12), the effects of PFHxDA (C16) on grip strength only appeared at the end of the recovery period. Hindlimb grip weakness was not necessarily accompanied by a low body weight. Further studies are required in order to clarify the mechanism responsible.

As for reproductive/developmental toxicity, the only effect observed was an inhibited postnatal body weight gain in pups at a maternal toxic dose of PFTeDA (C14). Similar results were observed in the study on PFHxDA (C16), but these changes were not significant. In our previous studies on long-chain PFCAs, postnatal body weight gain in pups was also inhibited at the highest dose (Hirata-Koizumi *et al.*, 2012; Kato *et al.*, in press; Takahashi *et al.*, 2014). In studies performed on PFDoA (C12) and PFOcDA (C18), such effects were accompanied by more severe reproductive/developmental effects, such as the deaths of dams at the end of pregnancy and stillbirths, and with more severe maternal toxic effects than those observed in the present study. The effect of long-chain PFCAs on postnatal development could be attributed to secondary effects due to maternal toxicity such as a low body weight during the lactation period. If PFTeDA (C14) reduced thyroid hormone levels as speculated above, it may be one cause of impaired postnatal development because Hapon *et al.* (2003) reported that hypothyroidism induced by a propylthiouracyl treatment impaired the growth of pups during the lactation period in rats. When the lipophilic properties of long-chain PFCAs (Inoue *et al.*, 2012) are considered, there is also the possibility that they were transferred via breast milk and affected the pups directly.

Based on the present results, the NOAELs for the repeated dose and reproductive/developmental toxicity were concluded to be 1 and 3 mg/kg/day for PFTe-

DA (C14) and 4 and 100 mg/kg/day for PFHxDA (C16), respectively. When the NOAELs were compared with those of PFUnA (C11), PFDoA (C12), and PFOcDA (C18) from our previous studies, the toxic potency of PFCAs was found to become weaker as the carbon chain length increased from C12 to C18 (Table 7). Since the previous comparative studies on the hepatic effects of PFCAs demonstrated increases in toxic potency due to an increase in the length of carbon chains up to C8 in rodents (Kudo *et al.*, 2006; Permadi *et al.*, 1993), the toxic potency of PFCAs was considered to be the strongest when the carbon length was C8 to C12. A clear chain length-dependent downward trend was observed in the renal elimination of PFCAs with a carbon chain length from C6 to C10 in rats (Ohmori *et al.*, 2003; Kudo *et al.*, 2001), and active renal tubular reabsorption via organic anion transport proteins was considered to be responsible for this (Han *et al.*, 2012). On the other hand, Wolf *et al.* (2008, 2012) reported that PFCAs of longer chain lengths induced more activity from mouse and human PPARα than those of shorter chain lengths up to C9 in transiently transfected COS-1 cells; therefore, not only toxicokinetic, but also toxicodynamic factors may contribute to the chain length-dependent toxicity of PFCAs with carbon chain lengths up to C8. Regarding PFCAs with carbon chain lengths of C11 and above, although no data is currently available to explain the cause of the chain length-dependent differences in toxic potencies, medium chain fatty acids (typically C6-C12) are known to be absorbed better from the gastrointestinal tract than long-chain fatty acids (typically longer than C12) (Ramirez *et al.*, 2001). Considering structural similarities, the gastrointestinal absorption of longer chain PFCAs may be poorer than that of PFCAs with shorter carbon chains. In order to clarify the cause of the differences in the toxic potencies of long-chain PFCAs, we are planning to first analyze serum PFCA levels in rats given different long-chain PFCAs.

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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# *Occurrence of 1153 organic micropollutants in the aquatic environment of Vietnam*

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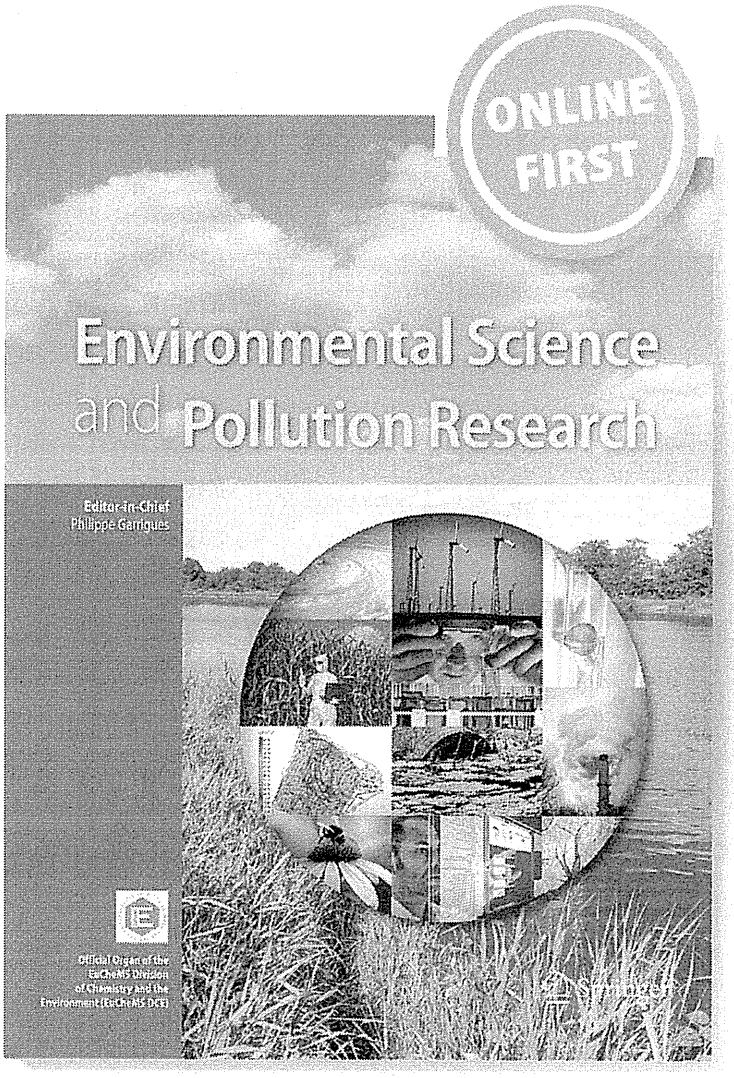


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# Occurrence of 1153 organic micropollutants in the aquatic environment of Vietnam

**H. T. C. Chau[1] · K. Kadokami[1] · H. T. Duong[1,2] · L. Kong[1] · T. T. Nguyen[2] · T. Q. Nguyen[2] · Y. Ito[1]**

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**Abstract** The rapid increase in the number and volume of chemical substances being used in modern society has been accompanied by a large number of potentially hazardous chemicals being found in environmental samples. In Vietnam, the monitoring of chemical substances is mainly limited to a small number of known pollutants in spite of rapid economic growth and urbanization, and there is an urgent need to examine a large number of chemicals to prevent impacts from expanding environmental pollution. However, it is difficult to analyze a large number of chemicals using existing methods, because they are time consuming and expensive. In the present study, we determined 1153 substances to grasp a pollution picture of microcontaminants in the aquatic environment. To achieve this objective, we have used two comprehensive analytical methods: (1) solid-phase extraction (SPE) and LC-TOF-MS analysis, and (2) SPE and GC-MS analysis. We collected 42 samples from northern (the Red River and Hanoi), central (Hue and Danang), and southern (Ho Chi Minh City and Saigon-Dongnai River) Vietnam. One hundred and sixty-five compounds were detected at least once. The compounds detected most frequently (>40 % samples) at μg/L concentrations were sterols (cholesterol, beta-sitosterol, stigmasterol, coprostanol), phthalates (bis(2-ethylhexyl) phthalate and di-*n*-butyl phthalate), and pharmaceutical and personal care products (caffeine, metformin). These contaminants were detected at almost the same detection frequency as in developed countries. The results reveal that surface waters in Vietnam, particularly in the center of large cities, are polluted by a large number of organic micropollutants, with households and business activities as the major sources. In addition, risk quotients (MEC/PNEC values) for nonylphenol, sulfamethoxazole, ampicillin, acetaminophen, erythromycin and clarithromycin were higher than 1, which indicates a possibility of adverse effects on aquatic ecosystems.

**Keywords** Screening analysis · Micropollutants · GC/MS · LC/TOF-MS · Pesticides · PPCPs

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## Introduction

Urbanization, industrialization, and intensive farming are having a negative impact on Vietnam's environment. As a result, surface water of rivers running through residential and industrial areas has been increasingly polluted by organic contaminants (Ministry of Natural Resources and Environment (MONRE) 2010). Untreated medical, industrial, and municipal wastewater are combined in municipal sewage systems and then discharged to canals and rivers. In particular, water pollution problems originating in domestic wastewater were clearly evidenced in large cities (MONRE 2010). For example, Ho Chi Minh City [HCMC, the most densely populated city in Vietnam (GSO 2013)], discharges 413,000 $m^3$ of wastewater per day, Hanoi discharges 155,000 $m^3$/day, and Hue-Danang discharge 58,800 $m^3$/day.

Water pollution was also found in rural or suburban areas of these cities. The main cause of water pollution in rural areas is pesticide and fertilizer residuals (Dang and Thiemann 2002;

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Anyusheva et al. 2012). Statistical data show that pesticide consumption rapidly increased from 66,000 t in 2005 to 124 000 t in 2012 (GSO 2013). Because of poor cropping practices, pesticides and fertilizers are often overused and enter waterways. High-density industrial development and agricultural activities in certain major river basins may also pollute rivers. For example, surface water of the Red River and Saigon-Dongnai River (SDR) is extensively used for irrigation, drinking, and cooking. Therefore, water pollution may affect large numbers of the population. The Red River is one of the main sources of water in northern Vietnam and has the second largest basin, covering 26 % of the area of Vietnam (MONRE 2006). Another important basin is that of the SDR; this basin encompasses the southeast principal economic zone including HCMC, Binhduong, Dongnai, and Baria-Vungtau provinces. These provinces comprise the most important industrial area in the country, with a high rate of economic growth.

In Vietnam, there have been few studies focusing on a small number of organochlorine pesticides, PCBs, PAHs, and others in surface or in sediments (e.g., Nhan et al. 2001; Dang and Thiemann 2002; Nguyen et al. 2007; Duong et al. 2008; Pham et al. 2010; Lamers et al. 2011). Owing to rapid economic growth and urbanization, monitoring of a large number of chemicals is needed to prevent expansion of environmental pollution. However, it is difficult to analyze such large numbers using existing methods because of the substantial time and expense involved with operating multiple definitive tests. We have developed novel screening methods that can measure hundreds of chemicals simultaneously (Jinya et al. 2013). In the present study, we applied the methods to river water in Vietnam and analyzed 1153 substances composed of 843 semi-volatile organic compounds (SVOCs) and 310 polar organic compounds (POCs), to elucidate the pollution status of the aquatic environment in Vietnam. From the results, a complete pollution picture of the aquatic environment in the country is portrayed.

## Materials and methods

### Materials

All solvents, *n*-hexane, acetone, and dichloromethane (DCM) for pesticide residue analysis, methanol of LC-MS grade, $Na_2HPO_4$, and $NaH_2PO_4$ were supplied by the Kanto Chemical Company (Tokyo, Japan). Reagents of target compounds and internal standards were purchased from Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemical Company, and Sigma-Aldrich (Tokyo, Japan). Purified water was obtained using a Millipore Milli-Q Advantage system (Nihon Millipore K.K., Tokyo, Japan).

### Water sample collection

All 42 samples were collected in March 2013. Fourteen samples were collected from the Red River (Fig. 1a, upstream to downstream). In Hanoi, three samples were taken in urban zones including the Kimnguu River (HN1), Lu River (HN2), and Tolich River (HN3). Other two samples (HN4, HN5) were collected from the Nhue River in a suburban zone of Hanoi (Fig. 1b). Figure 1c is for Hue (five samples, in an urban area HU4, and in a rural area HU1, HU2, HU3, HU5). Figure 1d is for Danang (seven samples, in an urban area DN3, DN7, and in a suburban area DN1, DN2, DN4, DN5, DN6). Four out of six HCMC samples were taken in the Thamluong (HCM7), Nhieuloc-Thinghe (HCM8), Logom (HCM9), and Tauhu (HCM10) canals (Fig. 1e), which appeared to be wastewater canals within urban areas. Since water from these canals has been collected and treated at wastewater treatment plants, water quality has improved (HCMC PC 2014). However, their surface water quality still does not meet national standards. Other two samples were taken from Anha (HCM6) and Kenhdoi (HCM11) canals in a suburban zone. For the SDR, three of five samples were collected from the Saigon River (HCM2, HCM3, HCM4) and one from the Dongnai River (HCM1); there was one other sample from the downstream of these two rivers (HCM5) (Fig. 1e). Detailed information and figures of sampling sites are given in Duong et al. (2015).

Surface water at the center of a stream was sampled from a bridge with a stainless steel bucket, which was pre-cleaned with solvents, purified water, and sample water. Each water sample was stored in a 1-L glass bottle previously washed with solvents and purified water. Bottles containing water samples were kept in an icebox and transported to our laboratory.

### Sample extraction and analysis

The GC-MS and GC-MS-MS analytical method for 950 SVOCs was undertaken according to the method of Jinya et al. (2013). A water sample (1 L), spiked with 1 mL of phosphate buffer (1 M, pH 7.0) to adjust the pH of each sample to 7, was fitted inside a vacuum manifold (3M Company, St. Paul, MN, USA) with flow rate less than 100 mL/min in a sequence with a glass microfiber disk (GMF 150, 47 mm, Whatman, Maidstone, UK), a styrene-divinylbenzene disk (Empore™ SDB-XD, 47 mm, 3M Co.), and an active carbon disk (Empore™ AC, 47 mm, 3M Co.). These disks were preconditioned by passing 10 mL of DCM, 10 mL of acetone, 10 mL of methanol, and 20 mL of purified water through them before use. After passing water sample through the disks, water remaining in the disks was completely removed using a vacuum for 30 min. The GMF and XD disks were eluted together with 5 mL of acetone (twice), followed by 5 mL of DCM. The AC disk was eluted with 5 mL of acetone (twice).

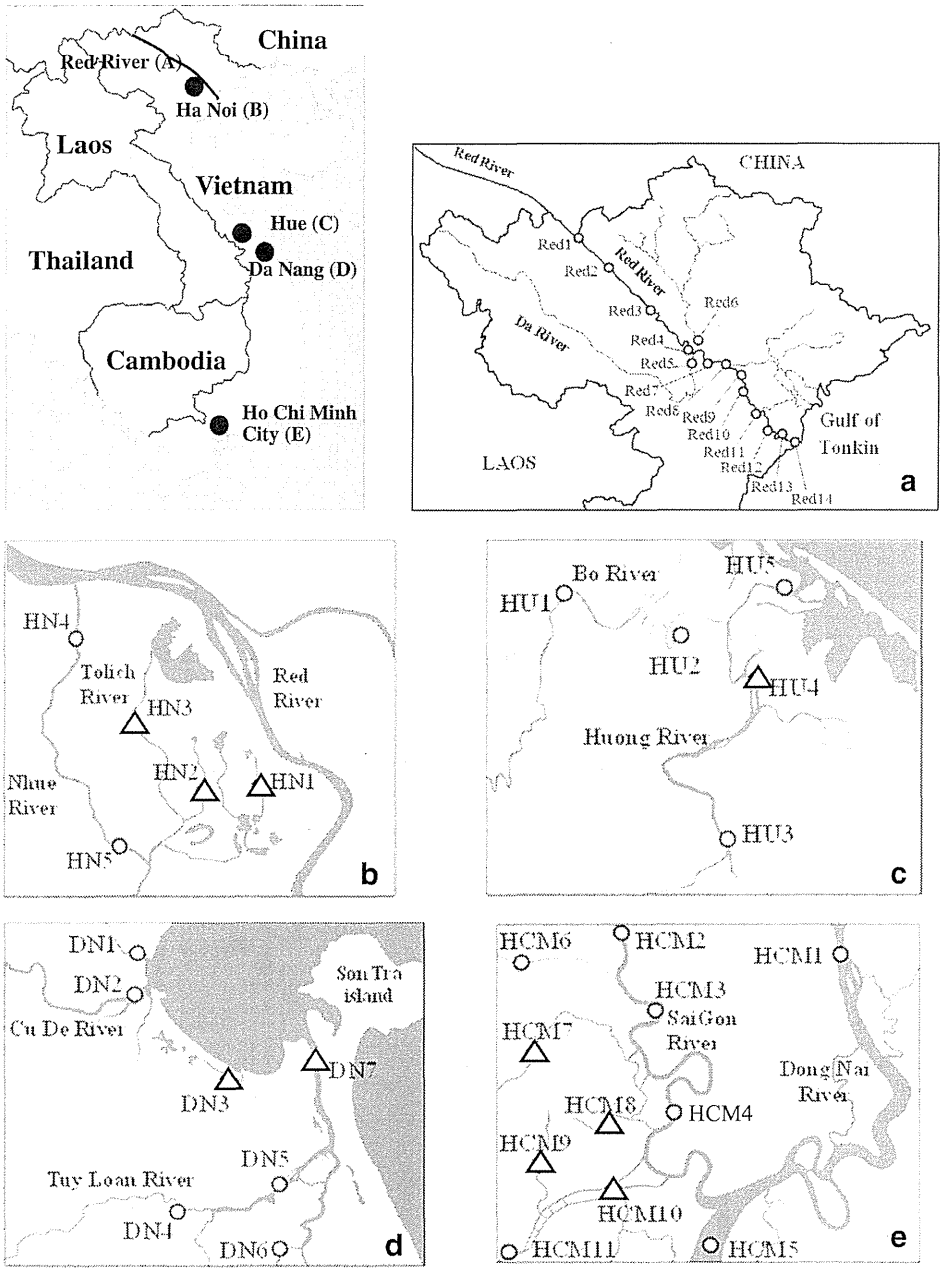


**Fig. 1** Location of 42 sampling sites. **a** Red River, **b** Hanoi, **c** Hue, **d** Danang, **e** Ho Chi Minh City and Saigon-Dongnai River. *Empty triangles* represent urban area; *empty circles* represent suburban area

The eluates were combined and concentrated into 1 mL with a nitrogen stream. The concentrate was diluted with 10 mL of hexane and dehydrated by adding $Na_2SO_4$ (preheated at 700 °C for 6 h). The dehydrated solution was concentrated to 1 mL, and then mixed internal standards (IS; 4-chlorotoluene-$d_4$, 1,4-dichlorobenzene-$d_4$, naphthalene-$d_8$, acenaphthene-$d_{10}$, phenanthrene-$d_{10}$, fluoranthene-$d_{10}$, chrysene-$d_{12}$, perylene-$d_{12}$) were added prior to instrumental analysis [GC-MS-SIM/Scan (QP-2100 Plus, Shimadzu, Tokyo, Japan) and GC-MS-MS-SRM (TSQ Quantum XLS, Thermo Fisher Scientific, Yokohama, Japan)].

For the analysis of 300 POCs, 1 mL of phosphate buffer (1 M, pH 7.0) was added to a water sample (500 mL) and filtered with a 1.2-μm glass fiber filter (Whatman, GF/C). Suspended solids (SS) were subjected to ultrasonic extraction with methanol twice. The filtrate was passed through a PS-2 Sep-Pak short cartridge (Waters Corporation) and an AC 2 Sep-Pak (Waters) using a Chratec Sep-Pak Concentrator (SPC 10-C; Chratec, Kyoto, Japan) with a flow rate of 10 mL/min, and then rinsed with 10 mL of purified water. The cartridges were then dried with nitrogen to remove water for 40 min. The cartridges were eluted with methanol (5 mL)

**Table 1** Concentrations (μg/L) of the chemicals found and the numbers of chemicals found (in parentheses)

| Group | Type of compound | Compound | *N* | Mean-max value of measured concentration (number of detected compound) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Red River (14 samples) | Hanoi (5 samples) | Hue (5 samples) | Danang (7 samples) | Saigon–Dongnai River (5 samples) | HCMC (6 samples) |
| Household chemicals | Leaching from tire | 2(3H)-benzothiazolone, 2-(methylthio)-benzothiazol, acetophenone, benzyl alcohol, phenylethyl alcohol | 5 | nd | 3.9–6.9 (4) | 0.043–0.087 (2) | nd | 0.0044–0.022 (1) | 3.3–12 (5) |
| | Petroleum | | 25 | 2.4–8.8 (22) | 23–37 (24) | 1.1–4.3 (22) | 4.1–8.0 (19) | 2.9–4.7 (21) | 33–100 (25) |
| | Plasticizers | Bis(2-ethylhexyl)phthalate, bisphenol A, butyl benzyl phtalate, di(2-ethylhexyl) adipate, diethyl phthalate, di-*n*-butyl phthalate, triphenylphosphate | 7 | 4.3–17 (7) | 14–22 (6) | 0.11–0.47 (3) | 1.3–5.1 (4) | 2.3–4.0 (7) | 13–38 (6) |
| | Disinfectants | 2-methylphenol, 3- and 4-methylphenol, phenol | 3 | 0.011–0.040 (1) | 15–28 (2) | nd | nd | nd | 17–63 (3) |
| | Others | 4-methyl-2,6-di-t-butylphenol; 4-*tert*-octylphenol; nonylphenol | 3 | 0.051–0.11 (1) | 3.7–7.0 (3) | 0.017–0.086 (1) | 0.020–0.044 (1) | 0.056–0.11 (2) | 7.2–28 (3) |
| Industrial chemicals | Intermediates | 2,4-dichloroaniline; 2-ethyl-1-hexanol; 2-phenylphenol; 3,4-dichloroaniline; 3,5-dimethylphenol; biphenyl; dicyclohexylamine; quinoline | 8 | 0.026–0.076 (1) | 3.9–5.4 (7) | 0.048–0.067 (1) | 0.40–1.8 (2) | 0.10–0.16 (1) | 4.2–21 (6) |
| | PAHs | 1,3-dimethylnaphthalene; 2,6-dimethylnaphthalene; 2-methylnaphthalene; fluoranthene; phenanthrene; pyrene | 6 | 3.2–44[a] (3) | 0.090–0.17 (3) | nd | 11–74[a] (3) | nd | 0.35–1.3 (4) |
| | PCBs | | 32 | 0.057–0.15[a] (2) | 1.8–5.3[a] (14) | 0.086–0.14[a] (1) | 0.18–0.35[a] (3) | 0.19–0.27[a] (2) | 1.7–7.6[a] (28) |
| | Paint/solvent | Isophorone | 1 | 0.010–0.14 (1) | 0.23–5.2 (1) | 0.069–0.26 (1) | nd | nd | 0.35–1.4 (1) |
| Pesticides | Fungicides | Azoxystrobin, carbendazim, cyprodinil, epoxiconazole, ethoxyquin, hexachlorobenzene, isoprothiolane, tricyclazole | 8 | 0.12–0.29 (2) | 0.14–0.21 (3) | 0.029–0.11 (3) | 0.17–0.35 (2) | 0.15–0.28 (4) | 0.15–0.22 (5) |
| | Herbicides | Acetochlor, alachlor, ametryn, atrazine, bensulfuron-methyl, butachlor, diuron, flufenacet, naproanilide, prometryn, siduron, tebuthiuron | 12 | 0.11–0.29 (4) | 0.16–0.34 (5) | 0.025–0.12 (3) | 0.13–0.90 (2) | 0.075–0.21 (4) | 0.54–1.2 (4) |
| | Insecticides | Acetamiprid; a-HCH; aldrin; carbofuran; *cis*-chlordane; trans-chlordane; dimethoate; fenobucarb; fenoxycarb; imidacloprid; o,p'-DDD; p,p'-DDD+o,p'-DDT; p, p'-DDE; permethrin 1; permethrin 2; piperonyl butoxide; promecarb; | 17 | 0.039–0.14[a] (3) | 1.8–2.9 (12) | 0.036–0.10 (3) | 0.034–0.22 (7) | 0.054–0.10 (5) | 1.0–3.0 (9) |
| | Sterols | Cholestanol, cholesterol, coprostanol, beta-sitosterol, stigmasterol | 5 | 5.3–17 (4) | 121–194 (5) | 3.8–6.5 (4) | 9.6–39 (4) | 8.6–11 (4) | 58–159 (4) |
| PPCPs | Antibiotics | Ampicillin, clarithromycin, erythromycin, griseofulvin, lincomycin, oleandomycin, roxithromycin, spiramycin, sulfadiazine, | 13 | 0.017–0.24 (3) | 3.7–5.5 (10) | nd | 0.12–0.86 (2) | 0.26–0.63 (2) | 2.1–4.4 (10) |

**Table 1** (continued)

| Group | Type of compound | Compound | *N* | Mean-max value of measured concentration (number of detected compound) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Red River (14 samples) | Hanoi (5 samples) | Hue (5 samples) | Danang (7 samples) | Saigon–Dongnai River (5 samples) | HCMC (6 samples) |
| | | sulfanilamide, sulfamethoxazole, sulfapyridine, trimethoprim | | | | | | | |
| | Other pharmaceuticals | Acetaminophen, atenolol, acetohexamide, antipyrine, caffeine, carbamazepin, cimetidine, cotinine, dicthyltoluamide, lidocaine, hexamethylenetetramine, L-menthol, losartan, metformin, nicotine, phenacetin, propranolol, sulpiride, testosterone, theophylline | 20 | 0.058–0.25 (4) | 22–38 (14) | 0.14–0.55 (2) | 0.27–1.5 (7) | 1.1–3.1 (8) | 17–60 (16) |
| Total number of detected compounds | | | 165 | 58 | 113 | 46 | 56 | 61 | 129 |

*N* number of detected compounds detected in all 42 samples at least once, *nd* not detected

[a] Concentrations were calculated in the unit of ng/L

and DCM (3 mL). After combining the eluates and the extract from SS, the mixture was concentrated to 50 μL and then spiked with 40 μL of three IS (5 μg/L, mixture of methomyl-$d_3$, pirimicarb-$d_6$, imazalil-$d_5$). The concentrate was diluted to 1 mL with purified water, filtered through a 0.2-μm syringe filter (Millex-LG) into an analysis vial, and subsequently measured by LC-TOF-MS.

### Analytical quality control

Method accuracy and precision were studied by recovery studies using surface water and effluent of sewage treatment plants spiked at different concentrations. The procedure blanks were analyzed every 6 samples to check for cross-contamination and interference.

For SVOC analysis, quality control measures were as described by Jinya et al. (2011, 2013). Two hundred-two SVOCs were selected as model compounds (MCs) having a wide range of physicochemical properties (structure, functional group, boiling points (145–536 °C)). The MCs included polycyclic aromatic hydrocarbons (PAHs), amines, alkyl phenols, halogenated phenols, phthalates, benzenes, alcohols, and some classes of pesticides. Recoveries were determined by analyzing purified and environmental sample spiked standards at two concentrations (0.1 and 0.5 μg/L). Most of the model compounds, which are representative of the target SVOCs, had recoveries of over 50 % (Jinya et al. 2013). Method detection limits (MDL) of chemicals measured by SIM and/or SRM were 0.0004–0.3 μg/L. The MDL of compounds measured by TIM were 0.005 to 0.5 μg/L.

For the polar substance analysis, the recoveries of 264 MCs from spike experiments at 0.05 and 0.2 μg/L were determined using purified water (replication $n=7$ for each level of concentration) and effluent wastewater ($n=5$) to be in the range 50–120 %. The relative standard deviation (RSD) values for recovery tests using purified water were in the range 3–25 % and the RSD of effluent samples between 5 and 30 %. Quantitation was performed by IS method using a peak area obtained at 100 V of fragmentor voltage. MDLs of POCs ranged from 0.008 to 0.4 μg/L. The correlation coefficients of calibration curves are higher than 0.99 for all the compounds analyzed.

## Results and discussion

### Detection of micropollutants in surface water samples

One hundred and sixty-five out of 1153 target compounds were detected at least once in surface water samples (Table S1). The total number of compounds found in Hanoi and HCMC samples were similar (113 and 129 compounds, respectively; Table 1), and two to three times higher than at

other sampling sites (Red River 58, Hue 46, Danang 56, SDR 61). Overall, the concentrations of substances detected in Hanoi and HCMC were much higher than in Hue, Danang, the Red River, and SDR (except for fungicides and herbicides; Fig. 2), because of differences in population density and economic activity. When comparing data from large cities and other sites, household chemicals, PAHs, and sterols had nearly identical numbers of detected compounds but vastly different total concentrations. The numbers and concentrations of fungicides and herbicides did not vary greatly between sites. PCBs, insecticides, and pharmaceutical and personal care products (PPCPs) were found in much higher numbers and concentrations in the large cities than at other sites (Table 1). When comparing the number and concentrations of detected organic compounds between urban and suburban area of cities, sampling sites in Hanoi urban area (HN1, HN2, and HN3) had high concentrations of household chemicals and PPCPs compared to those in suburban areas (HN4 and HN5; Fig. 2). This pattern was also observed among samples collected in urban area and suburban area of HCMC, Danang, and Hue (Fig. 2).

We screened 13 plasticizers; seven of these [bis(2-ethylhexyl)phthalate (DEHP), bisphenol A, butyl benzyl phthalate, di(2-ethylhexyl)adipate (DEHA), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), triphenylphosphate (TPP)] were detected in very high concentrations (μg/L level) at each sampling site. Maximum and average values of total detected concentrations were 38 and 13 μg/L in HCMC, 22 and 14 μg/L in Hanoi, and 17 and 4.3 μg/L in the Red River. DEHP was predominant, with high concentrations accounting for 71 % of the mean concentration of plasticizers detected in the Red River, 75 % in Hanoi, 76 % in SDR, and 65 % in HCMC.

The highest concentration of PAHs was 1334 ng/L (mean 64 ng/L), about three times lower than the value in a previous report from Vietnam (Duong et al. 2014) and about four times lower than in Tianjin, China (Kong et al. 2014). The number of detected PCBs (32) was similar to that reported by Duong et al. (2014), but their total concentrations were <7.6 ng/L

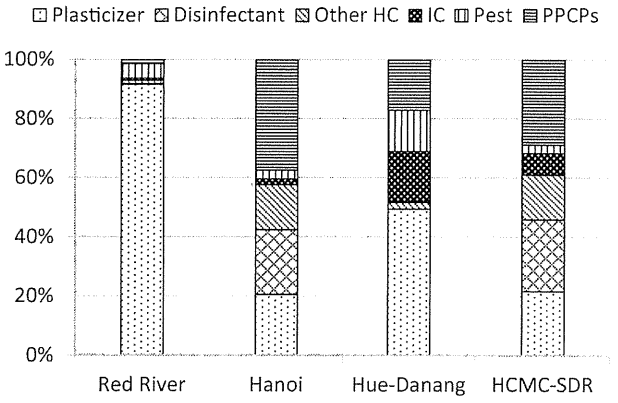


**Fig. 3** Percentages of concentrations of compounds detected at each location (*other HC*: other household chemicals; *ICs*: industrial chemicals)

(mean 0.54 ng/L), two times lower than previously reported values.

Only five out of the 12 sterols examined were observed and occurred at the highest concentration compared with the other compounds detected in this survey (Hanoi (194 μg/L), HCMC (159 μg/L)). A ratio of coprostanol/cholesterol ≥0.2 indicates sewage contamination (Grimalt et al. 1990). Generally, values near or greater than 0.2 were found in populous locations such as Hanoi (site HN1 0.96, HN2 0.86, HN3 0.88, HN4 0.37, HN5 0.82), Red10 (0.32, downstream of Hanoi), urban areas of Hue (HU4 0.19) and Danang (DN3 0.46), and HCMC (HCM6 0.30, HCM7 0.67, HCM9 0.79, HCM10 0.3, HCM11 0.20) (Table S1). Glassmeyer et al. (2005) suggested that a ratio exceeding 0.3 indicates fecal contamination. This means that wastewater containing feces from households was directly discharged into rivers or canals in urban areas, and domestic wastewater treatment plants were not operating effectively.

Thirty-three PPCPs were found in the survey, among which 13 compounds were antibiotics (ampicillin, clarithromycin, erythromycin, griseofulvin, lincomycin, oleandomycin, roxithromycin, spiramycin, sulfadiazine, sulfamethoxazole, sulfanilamide, sulfapyridine, and trimethoprim). The total

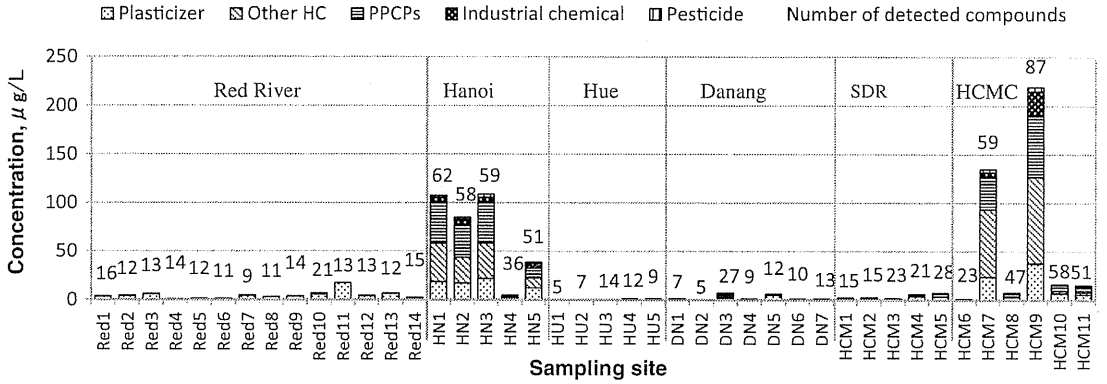


**Fig. 2** Concentrations and number of compounds detected at each sampling site

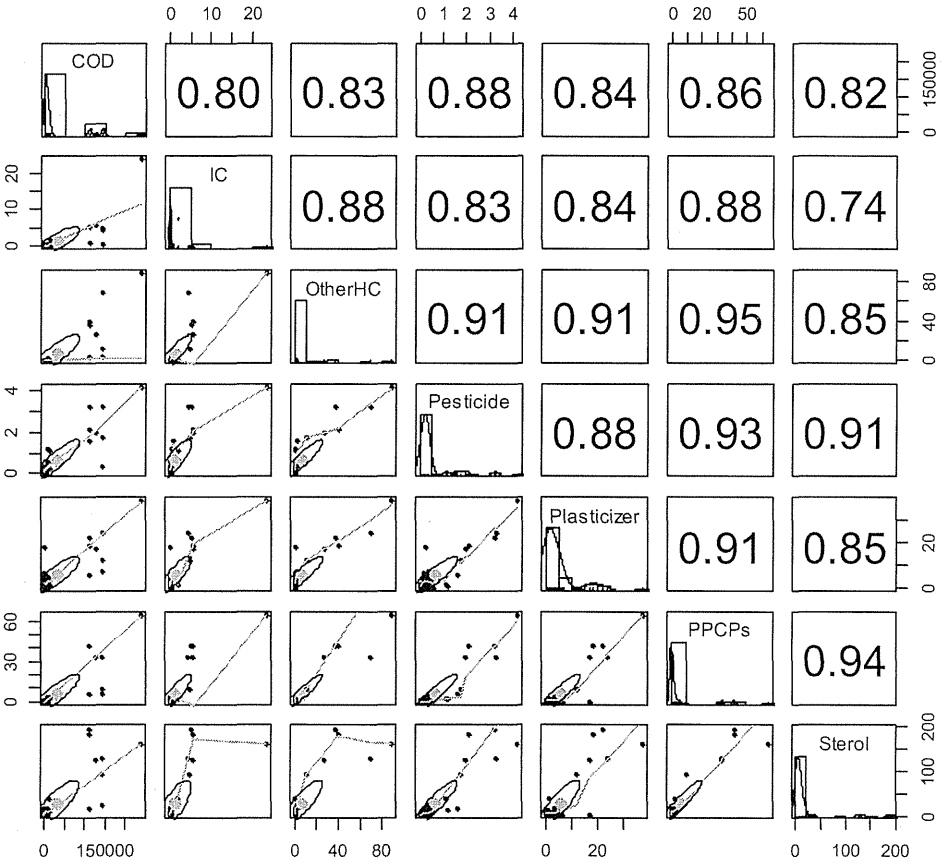


**Fig. 4** Correlation between groups of detected organic compounds (*COD* definition of chemical oxygen demand, *IC* industrial chemical, *Other HC* other household chemical)

concentration of all detected antibiotics was highest in Hanoi (5.5 μg/L; mean 3.7 μg/L), followed by 4.4 μg/L in HCMC (mean 2.1 μg/L). In Vietnam, antibiotics are dispensed without a doctor's prescription (Nguyen et al. 2011) and may enter the environment through feces or urine. However, it is possible that important point sources of antibiotics are hospitals because hospital wastewater contains high levels of antibiotics, and removal values through wastewater treatment plants are smaller than those in developed countries (Duong et al. 2008).

## Distribution of micropollutants in surface waters

More than 50 % of total micropollutant concentrations detected in both urban and suburban areas were household chemicals (Red River 92 %, Hanoi 58 %, Hue-Danang 52 %, HCMC-SDR 71 %; Fig. 3). The distributions of contaminants in the environment of Hanoi and HCMC-SDR were nearly identical but were very different to those of Hue-Danang and the Red River (Fig. 3).

Plasticizers are commonly used, and with millions of tons produced worldwide annually (Koch et al. 2003), these chemicals have become widespread in the environment (Fromme et al. 2002; Fauser et al. 2003). In the present study, plasticizers were a large proportion of detected contaminants, accounting for 21–22 % in Hanoi and HCMC-SDR, 50 % in Hue-Danang, and up to 91 % in the Red River. A likely source of plasticizers in the environment of large cities is storm water (Clara et al. 2010; Björklund et al. 2009). However, in the case of Hue-Danang and the Red River, untreated wastewater from craft villages is considered the main plasticizer source. Craft villages are classified into many different groups according to their products, such as textiles, construction materials, recycled metal, paper, or plastics. Most of these villages are in northern and central Vietnam, and the Red River basin has the largest number of craft villages, accounting for 60 % of all such villages in the country (MONRE 2008). All these villages have been facing environmental pollution problems. Pollution in these villages has not decreased and, in fact, has tended to increase. This may explain why industrial chemicals constituted large proportions of the contaminant composition in Hue-Danang (17 %).

Many pharmaceuticals and their metabolites have been detected in aquatic environments (Hereber 2002; Caliman and Gavrilescu 2009). In the present study, PPCPs contributed greatly to the total distribution; 37 % in Hanoi, 29 % in HCMC-SDR, and 17 % in Hue-Danang (Fig. 3). Because PPCPs are one of the major contaminants in surface samples

**Table 2** List of most frequently detected compounds in 42 samples

| Compound | Type of compound | LOD (ng/L) | Number > LOD | Number > 0.1 μg/L | Number > 1 μg/L | Number > 10 μg/L | Maximum (μg/L) | Median (μg/L) |
|---|---|---|---|---|---|---|---|---|
| Beta-sitosterol | Sterol | 100 | 42 | 42 | 31 | 7 | 25.2 | 1.98 |
| Cholesterol | Sterol | 100 | 42 | 42 | 34 | 8 | 70.6 | 1.66 |
| Stigmasterol | Sterol | 100 | 42 | 42 | 30 | 2 | 16.4 | 1.84 |
| PCB #1 | PCB | 0.03 | 35 | 0 | 0 | 0 | 0.32[a] | 0.11[a] |
| Dicyclohexylamine | Intermediate | 8 | 34 | 14 | 3 | 0 | 3.32 | 0.07 |
| Coprostanol | Sterol | 10 | 29 | 22 | 12 | 6 | 57.8 | 0.12 |
| 4-Methyl-2,6-di-t-butylphenol | Antioxidant | 25 | 28 | 7 | 0 | 0 | 0.41 | 0.04 |
| Bis(2-ethylhexyl)phthalate | Plasticizer | 10 | 27 | 26 | 24 | 6 | 19.0 | 2.25 |
| Cotinine | Nicotine metabolite | 8 | 27 | 10 | 5 | 0 | 2.84 | 0.01 |
| Di-*n*-butyl phthalate | Plasticizer | 10 | 26 | 19 | 7 | 0 | 4.92 | 0.08 |
| Triphenylphosphate | Plasticizer | 20 | 26 | 1 | 0 | 0 | 0.14 | 0.01 |
| p,p'-DDE | Insecticide | 0.03 | 25 | 0 | 0 | 0 | 4.14[a] | 0.04[a] |
| Di(2-ethylhexyl)adipate | Plasticizer | 10 | 24 | 14 | 0 | 0 | 0.44 | 0.03 |
| Atrazine | Herbicide | 10 | 24 | 0 | 0 | 0 | 0.03 | 0.01 |
| Lidocaine | Anesthetic/antiarrhythmic | 8 | 23 | 4 | 0 | 0 | 0.23 | 0.02 |
| Diethyl phthalate | Plasticizer | 10 | 22 | 15 | 6 | 0 | 7.49 | 0.03 |
| Bisphenol A | Plasticizer | 10 | 21 | 9 | 2 | 0 | 7.82 | 0.01 |
| Carbendazim | Fungicide | 8 | 19 | 9 | 0 | 0 | 0.21 | nd |
| Metformin | Antidiabetic | 8 | 19 | 13 | 7 | 0 | 8.25 | nd |
| Ethoxyquin | Fungicide | 8 | 18 | 6 | 0 | 0 | 0.29 | nd |
| Tricyclazole | Fungicide | 8 | 18 | 0 | 0 | 0 | 0.10 | nd |
| 4-*tert*-Octylphenol | Nonionic detergent metabolite | 10 | 17 | 3 | 0 | 0 | 0.85 | nd |
| Fenobucarb | Insecticide | 8 | 17 | 1 | 0 | 0 | 0.22 | nd |
| Caffeine | Food product | 10 | 17 | 16 | 8 | 1 | 13.0 | nd |

*LOD* limit of detection

[a] Calculated concentrations have units ng/L

of crowded cities, more research is needed on their fates and effects in the environment. Pesticides and industrial chemicals comprised only 3 and 2 % in Hanoi, and 3 and 7 % in HCM-SDR, respectively, or 2–7 times lower than the rates found in Hue-Danang.

## Correlations between organic compounds detected in surface waters

The water quality parameters pH, total suspended solids (SS), and chemical oxygen demand (COD) were measured in this survey (Table S1). COD was observed in the range from 0.32 to 240 mg/L. Seven sampling sites had COD values more than 5 times higher than Vietnam's 20 mg/L national surface water quality regulation (QCVN 08: 2008/BTNMT; HN1, HN2, HN3, HCM7, HCM9, HCM10, and HCM11). These sites are located in urban areas of Hanoi and HCMC. There were strong, positive correlations between COD and all groups of detected organic compounds (industrial chemicals, household chemicals, pesticides, plasticizers, and sterol; Fig. 4). Therefore, it can be said that there was no specific sources of contaminants, and surface water has become polluted by wastewater discharges from domestic, hospitals, factories, and agricultural activities.

## Most frequently detected compounds in surface waters

Twenty-four substances were found frequently (≥40 % samples, with detected concentrations >LOD; Table 2), including 4 sterols [beta-sitosterol, cholesterol, stigmasterol (100 %), and coprostanol (69 %)], 6 plasticizers [DEHP (64 %), DBP and TPP (62 %), DEHA (57 %), DEP (52 %), bisphenol A (50 %)], 6 pesticides [pp'-DDE (60 %), atrazine (57 %), carbendazim (45 %), ethoxyquin, tricyclazole (43 %), fenobucarb (40 %)], 4 PPCPs [cotinine (64 %), lidocaine (55 %), metformin (45 %), caffeine (40 %)], 2 industrial chemicals [PCB#1 (83 %), dicyclohexylamine (81 %)], and 2 household chemicals [4-methyl-2-6-di-t-butylphenol (67 %), 4-*tert*-octylphenol (40 %)]. The substances showing high concentrations (>1 μg/L) were sterols such as cholesterol (81 %), beta-sitosterol (74 %), stigmasterol (71 %), coprostanol (29 %), phthalate plasticizer of DEHP