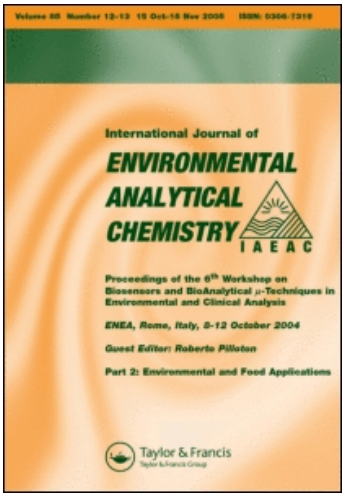

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Reducing Variability in Pollution Data: The Use of Lipid Classes for Normalization of Pollution Data in Marine Biota

K. Delbeke[a]; T. Teklemariam[a]; E. De La Cruz[a]; P. Sorgeloos[b]
[a] Laboratorium voor Ecologie, Brussels, Belgium [b] Artemia Reference Center, Gent, Belgium

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REDUCING VARIABILITY IN POLLUTION DATA: THE USE OF LIPID CLASSES FOR NORMALIZATION OF POLLUTION DATA IN MARINE BIOTA

K. DELBEKE*, T. TEKLEMARIAM*, E. DE LA CRUZ* and P. SORGELOOS**

**Laboratorium voor Ecologie, V.U.B., Pleinlaan 2, B-1050 Brussels, Belgium;*
***Artemia Reference Center, R.U.G., Rozier 44, 9000 Gent, Belgium*

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Organochlorine residues (PCBs and pesticides) are usually normalized on lipid content, gravimetrically quantified after extraction in organic solvents. In this study, the lipid content of different biotic groups (e.g. phytoplankton, shrimp, bivalves, fish), obtained through different types of extraction (total extraction versus specific extraction of apolar lipids) and quantification techniques (total gravimetric determinations versus specific determinations of lipid classes (Iatroscan)) were compared. The different lipid quantification methods were interrelated. An overestimation of lipid content due to non-lipid co-extracts was shown to interfere with gravimetric lipid quantifications. The relative contribution of these co-extracts depended on the type of sample studied. Differences in pollution levels (individual PCB congeners) between different biotic groups (phytoplankton, shrimp, cockles, fish) and between different fish tissues are discussed in relation to lipid content, determined by the different methods. An important reduction in the variability of pollution levels was noted when data were normalized on total neutral lipid content (Iatroscan). Highly significant linear regression parameters were obtained between the pollutant and the total neutral lipid content of marine biotic samples from the same water mass. Water mass specific bio-lipid contamination levels, characteristic for the whole biotic community (excluding seabirds and sea mammals), were determined for the different PCB congeners, normalized on total neutral lipid content. These bio-lipid contamination levels were related to the K_{ow} of the congeners. This functional relationship could be described by a second order polynomial regression.

KEY WORDS: Bioconcentration, PCBs, lipids, lipid classes.

INTRODUCTION

The impact of anthropogenic pollutants on humans and their environment are of great international concern. The large numbers and quantities of pollutants introduced into the environments necessitates the prediction of the pollutants' fate in the environment and their impact on biota and ecosystems. Therefore scientists study the mechanisms governing the pollutants' distribution in the environment, their biotic accumulation and their toxicity.

When reviewing the general literature on the fate of organic pollutants in aquatic ecosystems, one encounters two main approaches: the lipophilicity approach and the pharmacokinetic (or compartmental) approach. In the lipophilicity approach, the existence of a pseudo-equilibrium between the contamination of the biota and the surrounding water, is emphasized. Biotic contamination levels are dependent on the compounds' lipophilicity (partition coefficient octanol-water (K_{ow})) and water contamination levels. General relationships between the bioconcentration factor of the compounds in biota (fish) (K_b) and the compounds' partition coefficient octanol-water (K_{ow}) have been established, considering a large number of organic micro-pollutants[1,2,3,4]. These simple predictive relationships ($\log K_b = a \log K_{ow} - b$) have been further refined by introducing the compounds' MW and stereo-chemical structure[5,6] and including environmental variables (particulate and dissolved organic matter)[7,8,9,10] into the predictive models. The validity of the predictive models have been limited to compounds characterized by $\log K_{ows}$ of between 2 and 6 and to particles, cells and organisms having intensive interaction with the surrounding water mass, possibly through specific interfaces (e.g. gills). For birds and mammals, no intensive water-body interfaces exist and the organisms are therefore not directly in pseudo-equilibrium with the surrounding water. The lipophilicity approach has led to a general acceptance of normalization procedures for lipophile micro-organic pollutants, in the lower levels of aquatic biota, on the lipid content of the biota.

Monitoring data of micro-organic pollutants in aquatic biota collected from one water mass (excluding environmental variables) do, however, show large amounts of variability, not explained by the lipophilicity approach. This led to the so-called pharmacokinetic approach, in which the observed differences in contamination levels are described as being related to differences in uptake (through water and food), elimination, degradation and detoxification rates among individuals, sexes, species, etc.[11].

Although the two approaches seem conflicting, they are interdependent. Passive diffusion processes and physico-chemical partitioning of compounds between the different body compartments (blood plasma, blood cells, tissues . . .) are the basis of pharmacokinetic compartmental models[12,13]. Extrapolation of compartmental modeling from the level of the organism to the level of the aquatic ecosystem leads, on the other hand, to equilibrium partitioning between the organisms and the surrounding water. Therefore, the main question remains: how many compartments must one consider in order to understand the pollutants' behavior in aquatic ecosystems? Is it possible to understand and predict the contamination levels of organic pollutants in aquatic organisms by considering only two compartments (lipids and water) or must one consider differences in uptake, elimination, degradation and detoxification rates among the different cells, tissues, individuals and/or species of a specific aquatic ecosystem? The apparent contradiction between the lipophilicity approach (mainly derived from laboratory experiments) and the pharmacokinetic approach (mainly derived from field data) could be related to differences in data set variability (laboratory versus field data) and/or to field normalization procedures. In monitoring data, the normalization of organic pollutants is usually done on "total lipids", gravimetrically quantified. In natural aquatic environments, one can expect important differences in the lipid composition among tissues, individuals and species. These differences in lipid composition could interfere with organic pollutant data normalization on "total lipids". Schneider[14] indeed observed a lower

association of organochlorine residues with phospholipids compared to that with neutral lipids.

This study therefore aims to investigate the observed variations in micro-organic pollutant contamination levels of marine biota in relation to the analytical techniques used to measure "lipids" and in relation to specific associations between lipophile micro-organic pollutants and specific lipid classes.

MATERIALS AND METHODS

Different samples belonging to different environmental compartments (water; sediments; suspended matter; bivalves, *Spisula elliptica* and *Ensis arctuata*; shrimp, *Crangon crangon*; crabs, *Portunus depurator* and whiting, *Merlangius merlangus*) were collected with respectively a Niskin bottle, a Van Veen Grabber, a continuous centrifuge and a fishing net, on September 25th, 1992, at one station on the Belgium continental shelf (off Zeebrugge), with the R.V. "Belgica". The individuals of each species were mixed and homogenized, to obtain the so-called "mixed samples" (the crab carapace was removed) of sufficient size for lipid and organic pollutant analysis. The kidney, liver, gonads, muscle and gut of a large flatfish, *Pleuronectes platessa* (44 cm length), collected simultaneously, were removed. All samples were bubbled with N_2 and frozen (−18°C) on board.

The lipid content of the samples was quantified using different extraction and quantification methods. Neutral lipids were extracted (NLE) with acetone/hexane (1:9 v/v) as described in Delbeke *et al.*[15]; total lipids were extracted (TLE) in chloroform/methanol, as described by Ways and Hanahah[16]; neutral and total lipids were quantified gravimetrically as described in Delbeke *et al.*[15] and through the Iatroscan MK-5 TLC-FID system. In this latter system, silicagel SIII chromarods were used for the separation of lipid classes. A solvent system of hexane-diethylether-formic acid (85:15:0.04 v/v) was used for separation of neutral lipids[17]. The lipid classes were quantified using flame ionization detection (FID), operated with hydrogen (160 $ml.min^{-1}$) and air flow (200 $ml.min^{-1}$). The analyzer was connected to an Iatrocorder TC-11 Integrater plotter. Using external response factors (peak area) from composite standard mixtures, resembling the composition of the sample studied, this system measures different neutral lipids: cholesterol (Chol), cholesterol ester (Chol E), triglycerides (TG), free fatty acids (FFA) and polar lipids. The linear FID response for the different lipid classes ranged from 5 to 35 μg lipid loaded on the chromarod. Sample and standard applications were maintained in this linear response range. Recoveries, after addition of the standard mixture to the natural samples, ranged from 82–116%.

PCB extraction and clean-up procedures are described in Delbeke *et al.*[15]. The PCB congeners considered contain 3 to 8 atoms of chlorine per PCB molecule; they are characterized by Iupac nrs 28, 31, 52, 101, 118, 153, 138, 156, 170, 180 and 194. The PCB congeners were measured in a GC-ECD Interscience 8000 system, consisting of an automatic on-column injector; a two- dimensional capillary column system (60 meter DB 5 column and 60 meter DB1701 column) connected to 2 ECD detectors and a computerized integration system. Helium was used as carrier gas and nitrogen as detector gas; a temperature programme from 60°C to 280°C was used for peak separations. The congeners were

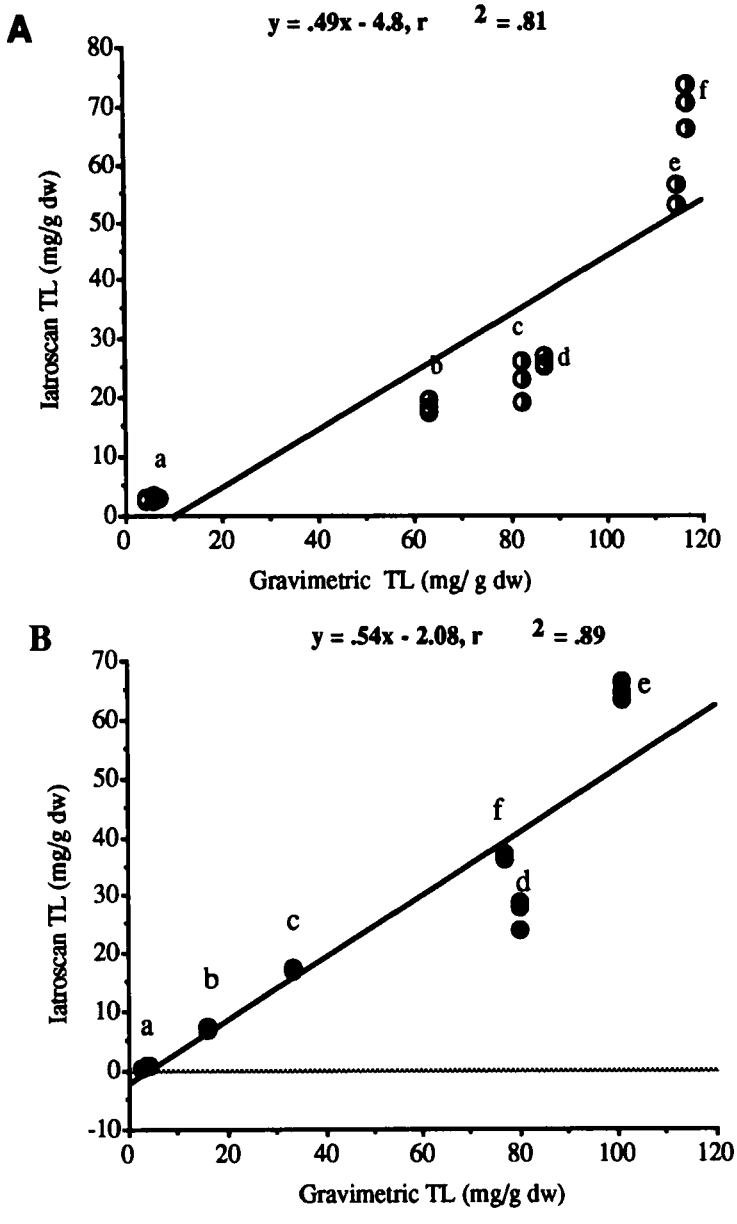

Figure 1 Comparison of gravimetrically determined lipid weights (neutral lipid extracts 1A and total lipid extract 1B) with the sum of the neutral lipids quantified with the TLC-FID Iatroscan system. a: suspended matter; b: *Spisula elliptica*; c: *Ensis arctuata*; d: *Crangon crangon*; e: *Portunus depurator* and f: *Merlangius merlangus*.

quantified through peak height response factors in their linear response range. Recoveries after addition of the standard mixture to the samples ranged between 86 and 119%. The set up was shown to be compatible in an ICES intercomparison exercise for standard solution, cleaned sediment and cleaned seal blubber samples.

RESULTS AND DISCUSSION

Comparison of lipid extraction and quantification techniques.

The lipid content of subsamples from different environmental compartments (sediments, suspended matter, *Spisula elliptica, Ensis arctuata, Crangon crangon, Portunus depurator* and *Merlangius merlangus*) which had been gravimetrically quantified after extraction of "neutral lipids" (NLE) and "total lipids" (TLE) were interrelated ($r=0.81$; $n=11$; $P<0.01$). The lipids extracted by the neutral lipid extraction (NLE) procedure accounted for 85% of the lipids extracted by the total lipid extraction (TLE) procedure.

Lipid data obtained through the Iatroscan quantification method were, for both extraction procedures, significantly lower than lipid data obtained through the gravimetric quantification method. The data sets were interrelated and Iatroscan quantification gave, on average, only 50% of gravimetric values (Figure 1A and 1B). Parish[18] and Sasaki and Capuzzo[19] reported Iatroscan/Gravimetric ratios of 0.84 and 0.88 for aquatic invertebrates. These values are higher than the mean value of 0.5 obtained in this study, considering different levels of the food chain, but more comparable to the ratio of 0.76, obtained here, for crabs only. The observed differences between Iatroscan and gravimetric lipid data were species dependent. The differences could partially be attributed to non-lipid co-extracts interfering with gravimetric determination as observed visually from the extract color, as observed from the initial non-lipid contamination peak (not interfering with lipid peaks) of the Iatroscan measurements and as discussed in e.g. Christie[20] and Fraser *et al.*[21].

The observed quantitative differences between the two extraction procedures (NLE and TLE) can be better understood by comparing the different lipid classes from different samples (sediments, mixed biota and flatfish tissues) after Iatroscan quantification (Table 1). Similar concentrations were observed for total neutral lipids and cholesterol. Polar lipids

Table 1 Comparison of lipid class quantification after neutral lipid (NLE) and total lipid extraction (TLE) procedures. n = number of samples; p = Level of significance of the difference between NLE and TLE (t-test)); NS = $p>0.05$.

Lipid class	*ratio: NLE/TLE*	*r(TNE-TLE)*	*n*	*p<*
Cholesterol ester	0.05	0.1	32	0.01
Triglycerides	0.38	0.93	32	0.01
Free fatty acid	1.50	0.58	32	0.05
Cholesterol	1.14	0.85	32	NS
Polar lipids	0.11	0.27	32	0.05
Total neutral lipids	0.88	0.54	32	NS
Total lipids	0.91	0.89	32	0.05

were, as expected[14], only extracted to a limited extent (11%) by the NLE procedure. The levels of free fatty acids were higher, while triglycerides and cholesterol ester values were lower in the NLE compared to the TLE. An auto-oxidation and/or hydrolyzes of triglycerides and cholesterol ester into free fatty acids, as described by Christie[20] and Kawai *et al.*[22] might explain this apparent anomaly. The NLE procedure, which has been developed for the analysis of stable compounds (organochlorine residues), is indeed subject to possible transformation. The procedure includes prolonged heating during Soxhlet extraction and several periods of exposure to air. Such transformations are minimized in the TLE procedure, a method specifically designed for lipid analysis.

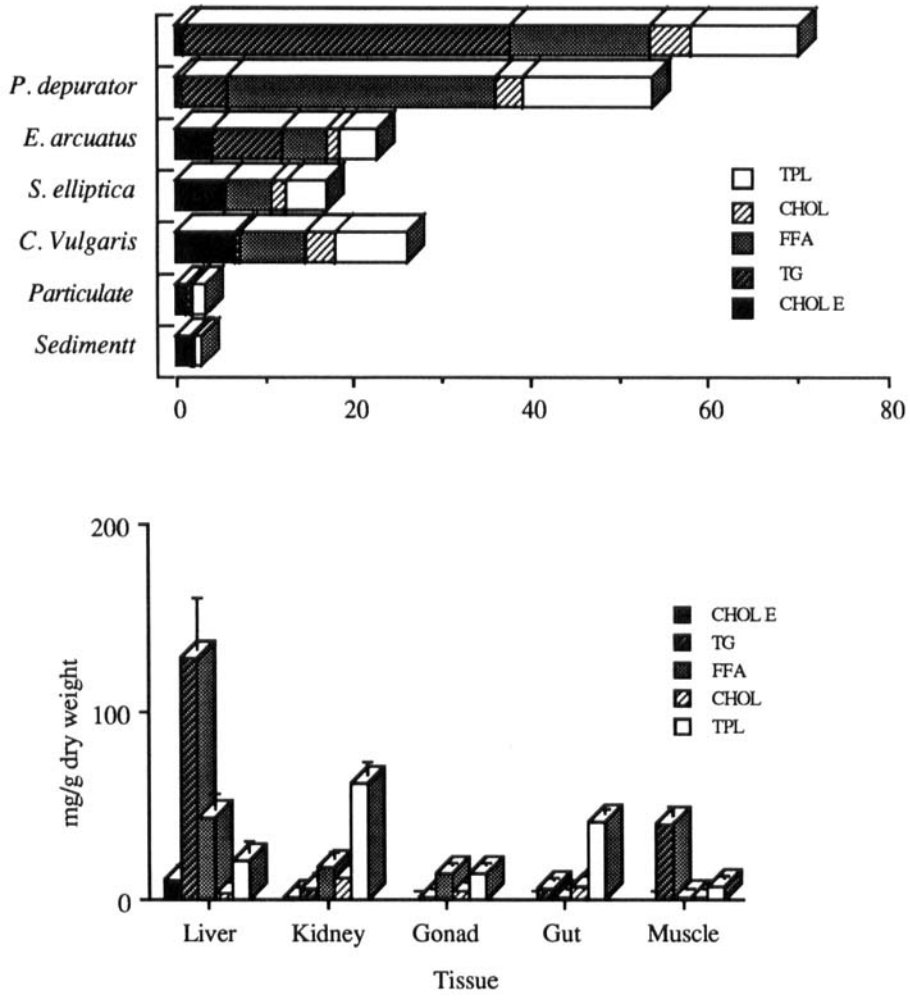

Figure 2 Lipid content (mg/g dry weight) and lipid composition of marine organisms (A) and tissues of *Pleuronectes platessa* (B). TPL: Total polar lipid; Chol: Cholesterol; FFA: Free fatty acids; TG: Triglycerides; Chol E: Cholesterol ester. C. Vulgaris = *Crangon crangon.*

Composition and quantification of lipids in different samples from the North Sea

The lipid pools in the different marine samples (sediments, mixed biota and flatfish tissues) were qualitatively and quantitatively compared using data from the TLE procedure and Iatroscan quantification.

In order to measure data reproducibility, 3 sub-extracts of each sample type were analyzed. The coefficients of variations varied between 2% (crab) and 24% (flatfish kidney). The whole data set had a mean coefficient of variation of 11%.

When comparing mean concentrations of lipid classes (Figure 2), it is clear that marine samples differed with respect to lipid composition. Triglycerides were especially abundant in *M. merlangus* and in the liver and muscle of *P. platessa*, free fatty acids were very important in *P. depurator* and polar lipids were important lipid constituents in flatfish kidney and gut.

PCB levels in different samples from the North Sea

Sample homogenicity and PCB data reproducibility was investigated in 7 subsamples of shrimp and in two replicates of the other samples. The weighted mean coefficients of variation for the different sample types were respectively for the IUPAC nrs 28:15%; 31:14%; 52:13%; 101:16%; 118:25%; 153:19%; 138:13%; 156:27%; 170:21%; 180:17% and 194:19%. Between 13 and 27% of the variation in PCB contamination was thus related to sample homogenicity and PCB data reproducibility with the data set.

When comparing PCB contamination levels on a dry weight base, an important increase in contamination from phytoplankton (14 ng/g dry weight) to bivalves (37 and 72 ng/g dry weight); to crustacea (49 ng/g dry weight) and crabs, feeding on small bivalve (190 ng/g dry weight) and finally to predaceous whiting (265 ng/g dry weight) and flatfish (83 to 902 ng/g dry weight, according to the tissue considered) could be observed.

Table 2 Correlation coefficients observed for mean PCB congener contamination levels and mean lipid levels in marine biota (suspended matter; *Spisula elliptica; Ensis arctuata; Crangon crangon; Portunus depurator* and *Merlangius merlangus*). The lipids are quantified gravimetrically after Neutral Lipid Extraction (NLE); gravimetrically after Total Lipid Extraction (TLE) and quantified as total neutral lipids by Iatroscan after Total Lipid Extraction. (n = 6; *, **, *** significance level of correlation coefficient < 0.05; 0.01; 0.001).

IUPAC nr	*TLE gravimetric*	*NLE gravimetric*	*TLE Iatroscan*
31	0.65	0.63	0.91*
28	0.76	0.75	0.93**
52	0.90*	0.82*	0.98***
101	0.65	0.45	0.83*
118	0.81	0.82	0.99***
138	0.79	0.89*	0.87*
153	0.77	0.78	0.97***
156	0.79	0.80	0.98***
170	0.75	0.78	0.98***
180	0.79	0.84*	0.98***
194	0.93**	0.93**	0.94***

Relationship between PCB congeners and lipid levels in marine biota.

PCB contamination levels (in ng/g dry weight) in the different biotic samples (mixed samples) were compared with their respective neutral lipid contents (in mg/g dry weight), extracted and quantified through the different methods: gravimetrically after Total Lipid Extraction (TLE); gravimetrically after Neutral Lipid Extraction (NLE) and quantified as total neutral lipids by Iatroscan after TLE. Important differences in the significance of the functional relationship between PCBs and lipids were observed when the different lipid quantification methods were compared (Table 2 and Figure 3 give some examples).

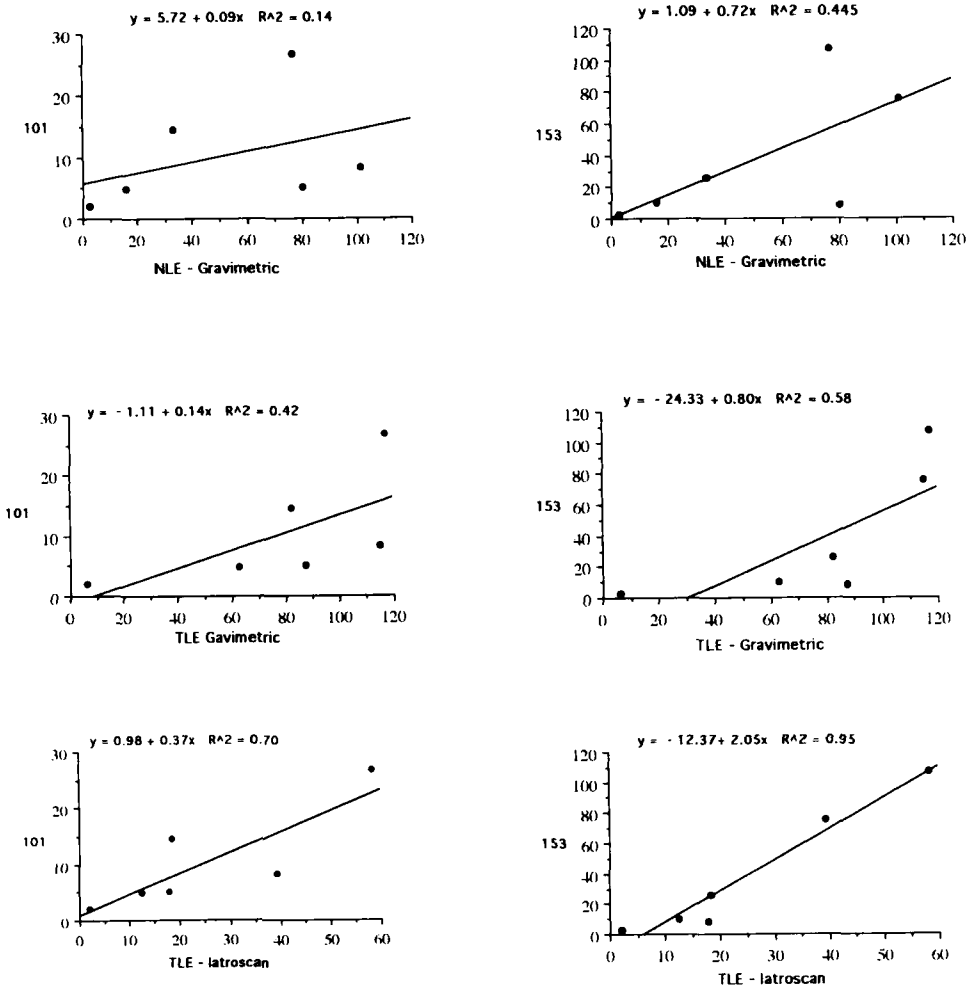

Figure 3 PCB congener levels (ng/g dry weight) in function of lipid levels (mg/g dry weight) quantified by the different quantification methods. Mean data for suspended matter; *Spisula elliptica; Ensis arctuata; Crangon crangon; Portunus depurator* and *Merlangius merlangus*. (n = 3 for lipids and n = 2 to 7 for PCBs). NLE = neutral lipid extraction procedure; TLE = total lipid extraction procedure.

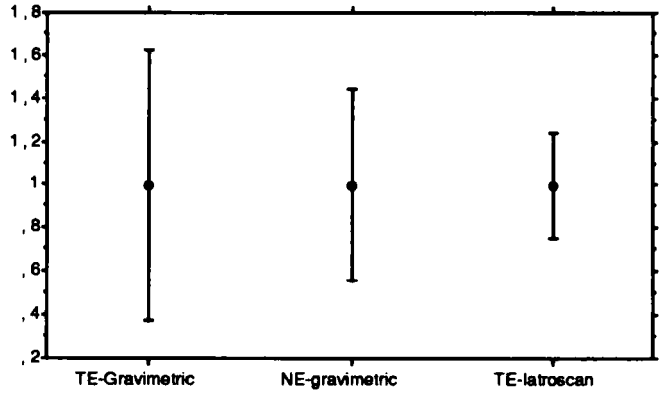

Figure 4 Relative variability in PCB congener contamination levels (as the sum of IUPAC nrs 28, 31, 52, 101, 118, 138, 156, 170, 180, 194) of different biotic samples (see Figure 3). The PCB levels are normalized on lipid levels quantified through different methods (TE = total lipids extract; NE = neutral lipid extract;).

The best fit was obtained when lipid content was expressed as total neutral lipids, quantified by Iatroscan. Similarly, the smallest variation in PCB data could be observed for PCB levels normalized on total neutral lipids (Iatroscan) (Figure 4). PCB levels in biota thus seemed to be significantly related to the total neutral lipid (TNL) content of the biota irrespective of the species considered or its ecological position in the marine ecosystem (Figure 5).

When the data for the flatfish tissues was included in the regression analysis, the observed relationship between PCBs and total neutral lipid levels could in general be extrapolated to flatfish tissues. As high lipid levels and correspondingly high PCB levels for the flatfish liver as well as a few outliers were observed, analysis of residuals was done. High leverage coefficients[23] were observed for liver lipid levels. Standardized residuals for the regression lines were calculated and multiplied by the leverage coefficients to obtain normalized residual values. These values were then compared with the mean normalized residual values to detect outliers (outside 95% confidence limits). Outliers, thus identified, were not considered in the final regression analysis (Figure 6). It should be noted that the analysis allowed inclusion of all data from mixed samples and from samples of flatfish kidney and gonad. The data for flatfish liver could, in spite of their high leverage coefficients, usually be included in the regression analysis. Gut and muscle were the two tissues having the highest unexplained residuals for some PCB congeners. This observation needs further investigation.

Because of the significant relationships between PCB congeners and lipid levels and the low regression intercept values, PCB contamination levels were normalized on total neutral lipids, quantified by the Iatroscan system. Bio-lipid contamination levels for the different biotic communities were compared (Figure 7) and the global bio-lipid contamination of the considered biotic community was investigated (Table 3).

The data from Figure 7 and Table 3 show differences in bio-lipid PCB levels among the different biotic samples. Nevertheless, they also show the relative reliability (50%) of the

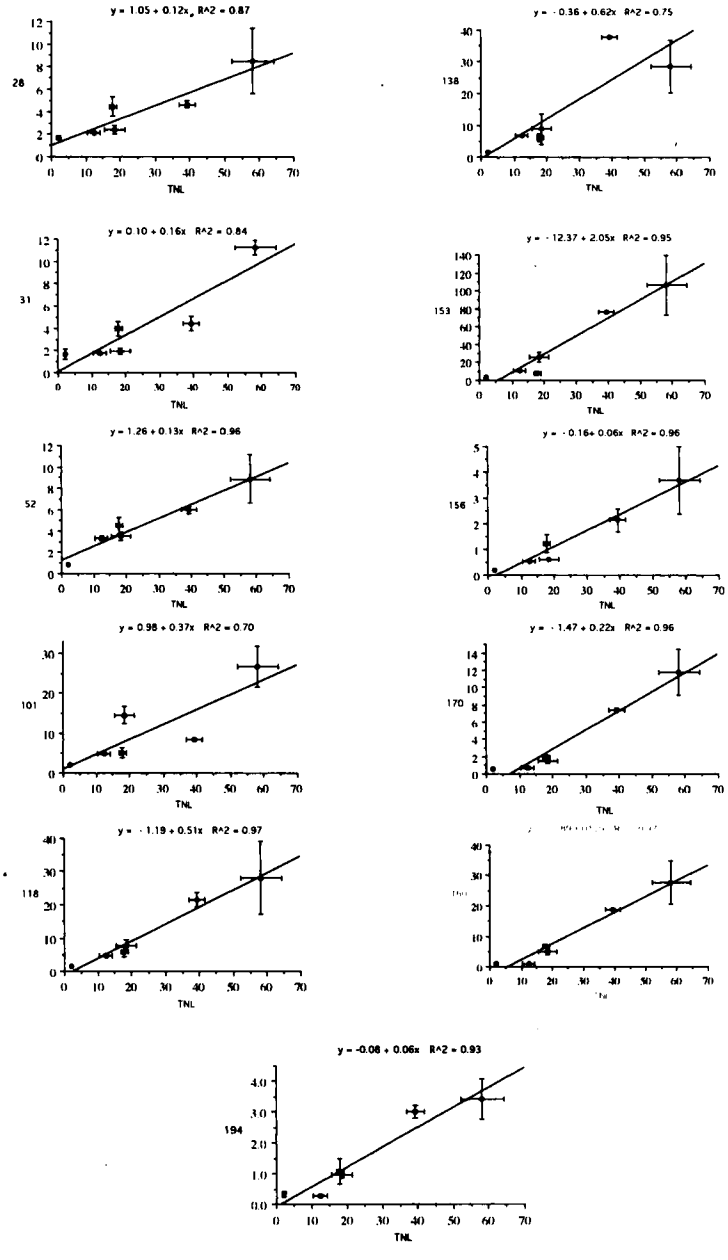

Figure 5 PCB congener levels (ng/g dry weight) in function of total neutral lipid levels (TNL) (mg/g dry weight) extracted as total lipids and quantified by Iatroscan. Mean data and sd for suspended matter; *Spisula elliptica; Ensis arctuata; Crangon crangon; Portunus depurator* and *Merlangius merlangus* (n = 3 for lipids and n = 2 to 7 for PCBs).

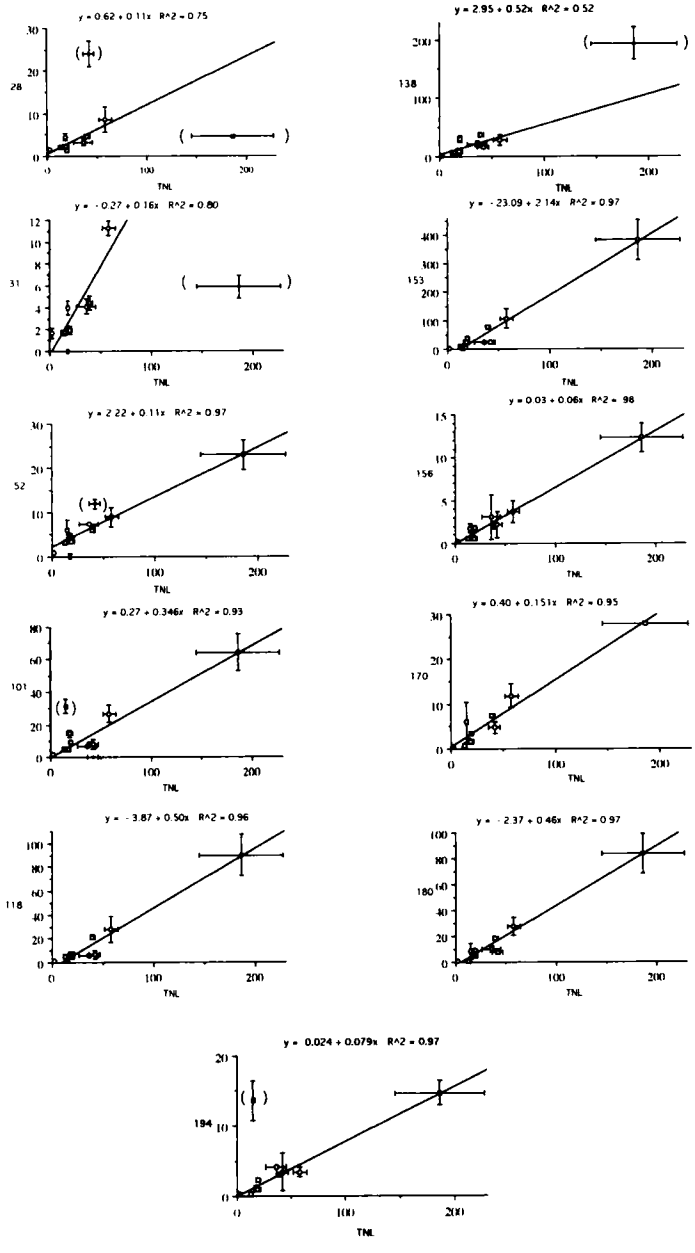

Figure 6 PCB congener levels (ng/g dry weight) in function of total neutral lipid levels (TNL) (mg/g dry weight) extracted as total neutral lipids and quantified by Iatroscan. Mean data and sd for suspended matter; *Spisula elliptica; Ensis arctuata; Crangon crangon; Portunus depurator; Merlangius merlangus and liver, muscle, gonad, gut, and kidney of Pleuronectes platessa.* ○: used for the regression analysis; ●: outliers not used for the regression analyzes (n = 3 for lipids and n = 2 to 7 for PCBs).

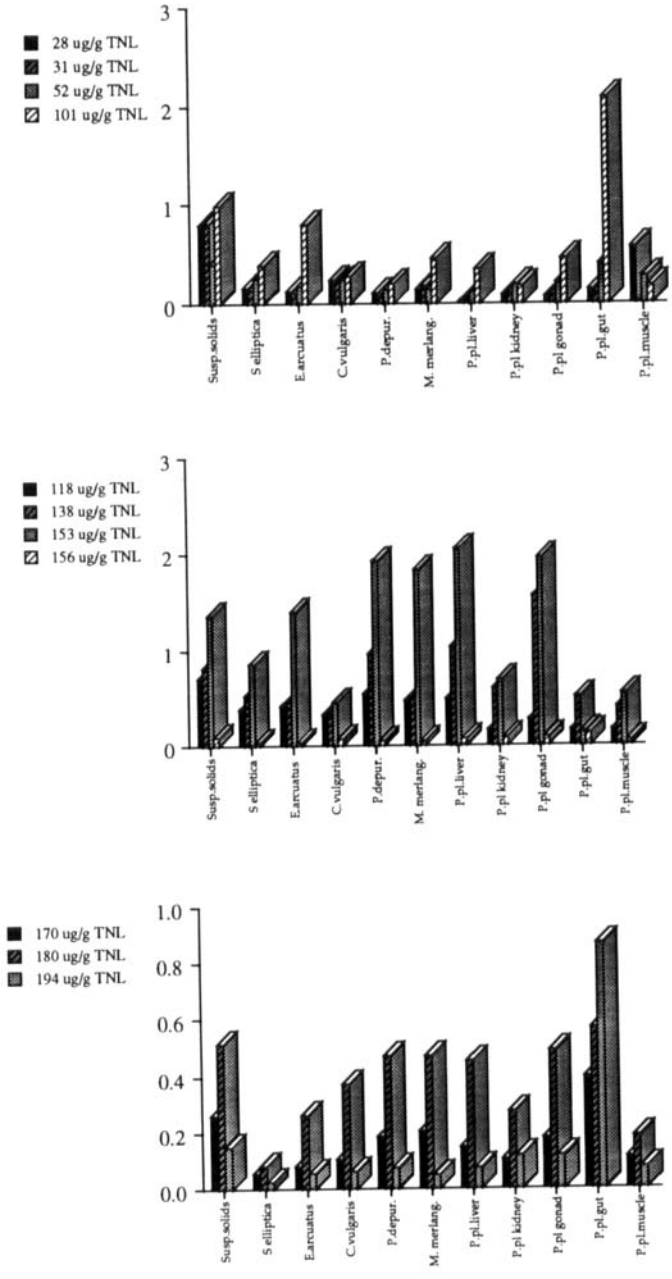

Figure 7 Comparison of mean PCB congener levels, normalized on total neutral lipids (TNL) among different biotic samples.

Table 3 Mean bio-lipid PCB concentrations of various biotic samples: suspended matter; *Spisula elliptica; Ensis arctuata; Crangon crangon; Portunus depurator; Merlangius merlangus* and tissues of *Pleuronectes platessa*) collected off Zeebrugge on 25 th September 1992. (TNL = Total Neutral Lipid; sd = standard deviation; cv = coefficient of variation; the outlying data, observed for some tissues of *Pleuronectes platessa* (see Figure 7 and text) were excluded from the mean values).

IUPAC nr.	*mean* μg/g *TNL*	*sd*	*cv*
28	0.23	0.26	123
31	0.23	0.28	117
52	0.24	0.11	46
101	0.45	0.31	69
118	0.38	0.19	51
153	1.21	0.68	56
138	0.74	0.37	50
156	0.07	0.03	40
180	0.38	0.16	42
170	0.17	0.10	58
194	0.08	0.04	52

estimated mean bio-lipid contamination level of the biota studied, at least for the higher chlorinated PCB congeners (4 chlorine atoms and more). The importance of the remaining variability in biotic PCB contamination levels normalized on total neutral lipids (50% and more for PCB congeners 28 and 31) still needs to be investigated further. It could be related to a need for data refinement and/or to differences in species specific accumulation due to food chain biomagnification as described by Connolly and Pedersen[24] or the influence of other water masses on the samples considered (swimming fish . . .).

A canonical correlation analysis using all the sample data revealed that within the lipid pool, cholesterol ester, triglycerides and free fatty acids explained most of the variability in PCB levels among the biotic samples. The more polar cholesterol and the polar lipids (as already mentioned by Schneider[14]) were associated very little with PCBs. However, no further reduction in data variability was observed with PCB normalization on the total amount of cholesterol ester, free fatty acids and triglycerides (compared to total neutral lipids). In the samples considered here, cholesterol played only a minor role in the neutral lipid pool and its variability (see Figure 2).

The relationship between biotic concentration factors and the physico-chemical characteristics of PCB congeners.

Bio-lipid concentration factors were calculated for each congener and each sample, using the biotic contamination levels expressed per total neutral lipid and the "dissolved contamination" of the water mass. We did realize that the procedure used to differentiate dissolved from particulate contamination levels (filtering on GFC filters) did not exclude very small particles nor the PCBs associated to dissolved organic matter present in the water column. It did however, provide an estimate of the so-called "dissolved PCB contamination". The

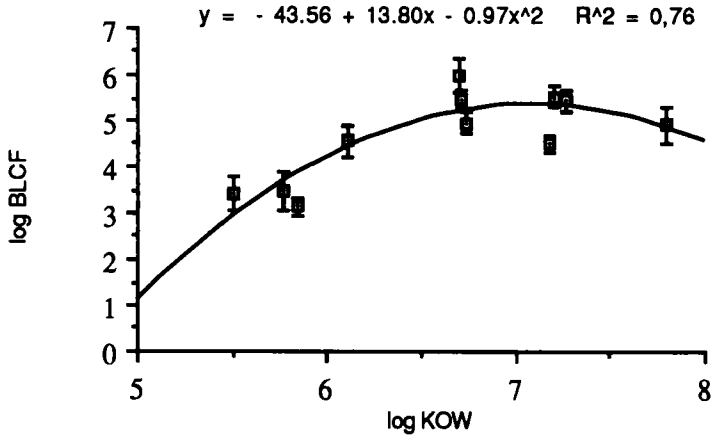

Figure 8 Bio -lipid concentration factors of PCB congeners in natural marine biota as a function of the K_{ow} of the congeners (n = the 11 different types of biotic samples (see Figure 6)). K_{ow} values are obtained from literature as follows: IUPAC nr 28 (25); 31 (26); 52, 118, 16, 170, 194 (27); 101 and 153 (28); 138 and 180 (29).

data showed, as described in the literature, that the lower chlorinated PCBs were relatively more important in the dissolved phase than the higher chlorinated PCBs.

The observed bio-lipid concentration factors ranged between log 2.8 and log 6.3. When comparing the mean log bio-lipid concentration factors for the different PCB congeners with their respective K_{ow}, the data were observed to fit a second order polynomial regression line (Figure 8). The form of the curve is similar to the ones described in the literature[5,6,30]. The leveling off of the curve has been related to a reduction in the bioavailability and a decrease in assimilation efficiency at higher Kow[24,30]. Shaw and Connel[6] proposed that spheric equivalency factors be introduced to improve linearity but this method did not have the desired result for our dataset.

The observed relationship is, however, limited to the water mass considered, no extrapolation to other stations or times can be made as no attempt was made in this study to introduce environmental factors such as particle concentration effect[7,8,9,10] or the amount of dissolved organic matter[31]. Extrapolation to other water masses and other organic pollutants should, therefore, still be investigated.

CONCLUSION

Normalization of PCB levels on total neutral lipids, extracted as total lipids and quantified by Iatroscan largely reduces the variability in PCB pollution data in marine biota. Its use could lead to better comparison of data among scientists and a reduction in the costs of monitoring programmes. The normalization procedure proposed here allowed better extrapolation among biota from the same water mass, although important interspecies differences are still observed.

Further research (especially considering other biotic species and other organic pollutants) could lead to a more general insight in the existing relationships between lipid classes and organic pollutants. To further predict and assess pollution by organic compounds in marine biota, field data concerning the influence of environmental factors (organic matter, salinity, temperature) should be collected.

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