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ORGANOCHLORINE COMPOUNDS (POLYCHLORINATED BIPHENYLS AND PESTICIDES) AND POLYCYCLIC AROMATIC HYDROCARBONS IN POPULATIONS OF *HEXAPLEX TRUNCULUS* AFFECTED BY IMPOSEX IN THE LAGOON OF VENICE, ITALY

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Abstract—Concentrations of polychlorinated biphenyls (PCBs), organochlorine pesticides, and polycyclic aromatic hydrocarbons (PAHs) were measured in gastropods from the Lagoon of Venice, Italy. The visceral coil and the rest of the soft body of organisms (*Hexaplex trunculus*) sampled at two stations inside the lagoon and three stations on the seaward side were analyzed to evaluate their contamination levels. Preferential accumulation of PCBs and pesticides in the visceral coil (>80%) compared with the rest of the soft body was observed, whereas on average, PAHs showed no preferential partitioning. Differences between levels of organochlorine contaminants in the gastropods highlighted a gradient of pollution from the stations inside the lagoon (PCBs, 45–363 ng/g; pesticides, 4–51 ng/g) to the sea (PCBs, 13–131 ng/g; pesticides, 2–29 ng/g). The possible role of the three classes of contaminants, in addition to that of organotin compounds (OTCs), previously analyzed in the same samples, in causing one of the anatomic modifications because of imposex in this gastropod also was studied. A modeling approach by partial least squares (PLS) in latent variables was applied to explain the penis length of imposex-affected females with concentrations of organic pollutants. The synergistic role of PCBs, pesticides, and OTCs was evidenced, whereas the contribution of PAHs appeared to be very low.

Keywords—Polychlorinated biphenyls Pesticides Polycyclic aromatic hydrocarbons *Hexaplex trunculus* Imposex

INTRODUCTION

Polychlorinated biphenyls (PCBs), organochlorine pesticides, and polycyclic aromatic hydrocarbons (PAHs) are classified as persistent organic pollutants because of their long-lasting presence in the environment, tendency to bioaccumulation, and toxicity to organisms. Polychlorinated biphenyls have been used in several industrial applications (e.g., dielectric fluids in transformers and capacitors) thanks to their chemical and thermal stability, nonflammability, high boiling point, high viscosity, and low vapor pressure. Organochlorine pesticides, intentionally released at their point and time of application, have been used in agriculture to protect crops from insects. In addition, one type of organochlorine, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), was used as an insecticide to fight and prevent insect-borne diseases like malaria.

Polycyclic aromatic hydrocarbons may be formed by various natural processes, such as high-temperature pyrolysis of organic material, low to moderate temperature diagenesis of sedimentary organic material, and direct biosynthesis by microbes and plants. Nevertheless, a wide variety of human activities, including combustion of oil, wood, fossil fuels, or organic matter, is continuously increasing the load of PAHs in the environment. Because of their slow biodegradation, PCBs and DDT are still present in the environment even though their use was discontinued in the 1980s and 1970s, respectively. Recently, the presence of PCBs, PAHs, and organochlorine pesticides in sediments [1,2] and seaweeds [3] of the Lagoon of Venice, Italy, has been reported.

An increasing amount of experimental evidence has demonstrated that PCBs, organochlorine pesticides, and PAHs can act on the endocrine and reproductive systems of humans and animals and caused these pollutants to be included in the list of endocrine disruptors [4–9]. The U.S. Environmental Protection Agency has defined endocrine disruptors as ''exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior'' (http://www.epa.gov/endocrine/Pubs/smithrep.html). They include a large and still-increasing number of natural and anthropogenic chemicals with various chemical structures [4].

One of the best-known hormonal disturbances in invertebrates is the phenomenon called imposex, a term coined by Smith [10] to indicate the overlap of secondary male sexual features, such as the penis and vas deferens, in females of several gastropod species. The detailed biochemical mechanism of this phenomenon remain unclear, but a number of hypotheses have been suggested [11–14], all involving the action of pollutants on the endocrine system. Although the environmental evidence shows tributyltin (TBT) as the main causal agent of imposex in marine invertebrates [15–18], other pollutants have been shown to induce this phenomenon: Triphenyltin (TPhT) causes the same effect as TBT in the gastropods *Thais clavigera* (Kuster, 1858) and *Thais bronni* (Dunker, 1860) [19] and induces imposex in the freshwater ramshorn snail, *Marisa cornuarietis* (Linnaeus, 1758) [20]. It also has been suggested that copper and environmental stress induce the development of imposex in *Lepsiella vinosa* (Lamark, 1822) [21].

In a previous study [22], a significant relationship, mathematically interpreted in the form of a sigmoidal curve, was

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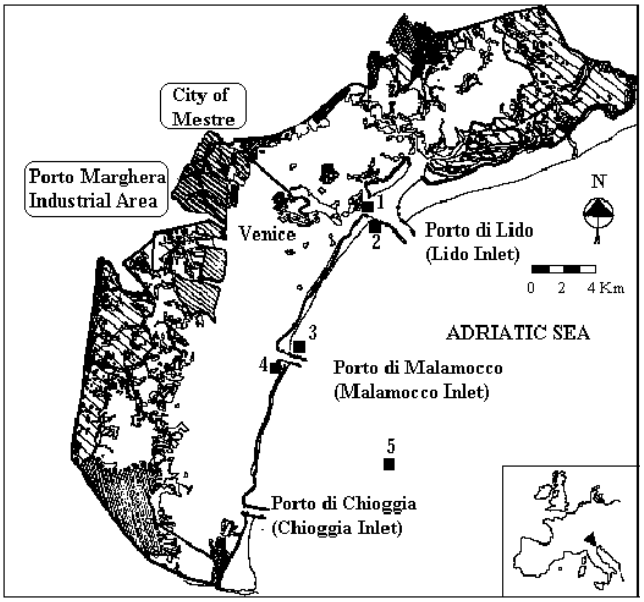

Fig. 1. Lagoon of Venice (Italy) and location of sampling stations (1 = S. Nicolò del Lido; 2 = Lido Mare; 3 = Alberoni; 4 = S. Maria del Mare; 5 = Chioggia).

found between organotin concentrations and penis length of female *Hexaplex trunculus* (Linnaeus, 1758) (Gastropod, Muricidae) from the Lagoon of Venice. Nevertheless, the variance of penis length was not explained completely by organotin concentration, indicating the possible contribution of other substances in the induction of this anatomic modification.

In the present study, other endocrine disruptors, such as PCBs, organochlorine pesticides, and PAHs, were analyzed in the same tissues of *H. trunculus* to verify their possible synergic role together with TBT in promotion of the anatomic modifications induced by imposex. Additional aims of the present work were to study the partitioning of these pollutants in the visceral coil and the rest of the soft body of the gastropods for insights regarding how these organisms handle these kinds of pollutants, to measure the extent of organism contamination because of various pollutants at five representative stations of the Lagoon of Venice, and to analyze the congener pattern for each class of pollutants, identifying the more bioaccumulable congeners.

MATERIALS AND METHODS

Sampling

From 40 to 60 organisms of the gastropod *H. trunculus* were sampled during a period of one year starting from January 2002 at five stations: Two inside the Lagoon of Venice, and three outside the lagoon. (Fig. 1). The two stations inside the lagoon were S. Nicolò del Lido and S. Maria del Mare. The station S. Nicolò del Lido is located near the port of Lido and is influenced both by the industrial zone of Porto Marghera and the agricultural areas inland from the lagoon. The station S. Maria del Mare is located on the Malamocco inlet, through which cargo ships and oil tankers enter the lagoon and, moving along the Malamocco–Marghera Canal, reach the industrial area of Porto Marghera. The other three stations are Alberoni, Lido Mare, and Chioggia. Alberoni is located on the seaward side of the Malamocco inlet, not far from the station of S. Maria del Mare. Lido Mare is close to the Lido inlet, through which tourist and commercial ships as well as pleasure craft enter the lagoon. Chioggia is located in the sea, 5 miles off the port of Chioggia, and serves as a reference site.

Random sampling was performed manually at all stations during low tide except at Chioggia, where a trawl was necessary. After sampling, the organisms were transported to the laboratory and stored at −20°C until analysis.

Biological analysis

After thawing, each shell was measured with a caliper and cracked in a vice; the organism was extracted and the mantle longitudinally cut to determine the sex by observing the presence or absence of a vaginal opening and a capsule gland, features that are present only in normal and imposexed females. The stage of imposex was determined according to the vas deferens sequence (VDS) proposed for *H. trunculus* [23], and male and female penis lengths were measured to the nearest 0.1 mm. The VDS Index (VDSI), or mean of all imposex stages recorded in a population, and relative penis size index (RPSI), or the percentage ratio between the cubed average female penis length and that of the male, were calculated for each population of *H. trunculus* according to the method described by Gibbs et al. [18].

From two to six organisms with the same sex and the same (when possible) or similar stage of imposex were pooled, separating the visceral coil from the rest of the soft body. Every pool of tissues was freeze-dried, homogenized, and stored at −20°C until chemical analysis.

Polychlorinated biphenyls, organochlorine pesticides, and PAHs

In the analytical procedure used, only one step is required for both cleanup of extracts and simultaneous separation of chlorinated and nonchlorinated hydrocarbons [24].

Tissues (0.5 g of visceral coil and 2 g of the rest of the soft body) were extracted three times in an ultrasonic bath for 2 h with 25 ml of *n*-hexane/dichloromethane (4:1) each time. The three aliquots were combined and the volume reduced to 1 ml in a rotary evaporator.

A chromatographic column (length, 45 cm; width, 1 cm) was used to clean up samples and achieve separation of the three classes of compounds. The column was filled with *n*-hexane/dichloromethane (4:1) and packed, in the following order, with silica gel (6 g), alumina (8 g), florisil (1.5 g), anhydrous sodium sulfate (1 g), copper (1 cm), and anhydrous sodium sulfate (1 g) again. Before column packing, silica gel, alumina, florisil, and sodium sulfate were cleaned with dichloromethane, dried, activated for 16 h at 250°C, and cooled under vacuum. Alumina, deactivated with 120 μl of distilled water, and silica gel also were sonicated for 2 h with *n*-hexane/dichloromethane (4:1). Copper was activated with HCl and washed with methanol and *n*-hexane/dichloromethane (4:1).

After elution of 30 ml of *n*-hexane, the extract was introduced into the column and the three classes of compounds separated by eluting various amounts of solvents: 20 ml of *n*-hexane to elute alkanes in the first fraction, 80 ml of *n*-hexane to elute PCBs and pesticides in the second fraction, and 75 ml of *n*-hexane/dichloromethane (3:2) to elute PAHs and the remaining pesticides in the third fraction. Only the second and third fractions were analyzed. They were reduced to a volume of approximately 500 μl in a rotary evaporator (Büchi, Flawil, Switzerland), transferred to vials, and after evaporation overnight to dryness, redissolved with 200 μl of iso-octane and stored at −20°C until instrumental analysis.

Instrumental analysis

The extracts containing PCBs and organochlorine pesticides (second and third fractions) were injected in a gas chromatograph coupled with an electron-capture detector (Hewlett-Packard 5890 series II, Wilmington, DE, USA). The fractions containing PAHs (third fractions) were injected into a gas chromatograph with a high-resolution capillary column (Hewlett-Packard 5890 series II) coupled to a low-resolution mass spectrometer (Hewlett-Packard 5970 B). The second and third fractions also were injected into a gas chromatograph–mass spectrometer to confirm the presence and quantification of PCBs and pesticides.

Gas chromatograph–electron-capture detector operative conditions and temperature programs for PCB and pesticide determinations were as follows: Capillary column, HP-5 (5% phenyl methylsiloxane; inner diameter, 0.20 mm; length, 50 m; film thickness, 0.33 μm); injector temperature, 250°C; splitless injection; temperature program, 110°C for 1 min, 9°C/min up to 141°C, 3°C/min up to 280°C, postrun for 5 min at 280°C; carrier gas, helium; detector temperature, 300°C.

Gas chromatograph–mass spectrometer operative conditions and temperature programs for PAH determination were as follows: Capillary column, HP-5 (5% phenyl methylsiloxane; inner diameter, 0.20 mm; length, 50 m; film thickness, 0.33 μm); injector temperature, 300°C; splitless injection; temperature program, 60°C for 1 min, 18°C/min up to 140°C, 10°C/min up to 252°C, 14°C/min up to 300°C, postrun for 22 min at 300°C; carrier gas, helium; transfer line temperature, 280°C.

Gas chromatograph–mass spectrometer operative conditions and temperature programs for PCB and pesticide confirmations were as follows: Capillary column, HP-5 (5% phenyl methylsiloxane; inner diameter, 0.20 mm; length, 50 m; film thickness, 0.33 μm); injector temperature, 300°C; splitless injection; temperature program, 110°C for 1 min, 12°C/min up to 141°C, 5°C/min up to 280°C, postrun for 18 min at 280°C; carrier gas, helium; transfer line temperature, 280°C.

Mass spectrometer detection was performed by electron-impact ionization (70 eV) in the selected-ion monitoring mode.

Quantification was performed with the internal standard method. Internal standards were as follows: PCB 30 for PCBs; a mixture containing naphthalene-d_8, phenanthrene-d_{10}, and perylene-d_{12} for PAHs; and pentachloronitrobenzene for pesticides. Response factors were calculated monthly through a calibration curve of six to seven standard mixtures covering the entire range of concentrations examined. Quality control of the analytical data also was performed: Analysis of blanks excluded potential contamination during the analytical procedure, and spiking experiments verified recovery yields. The limits of detection of each congener, calculated as 3σ of blank concentration, were lower than 0.1 ng/g for PCBs, lower than 0.06 ng/g for pesticides, and lower than 7 ng/g for PAHs.

All samples were analyzed in duplicate, and all concentrations reported in this work, expressed as nanograms of compound per gram of dry tissue (ng/g), are the average of the two replicates. The error of each determination was calculated as the half-variation interval of the measures and was always within 15%.

The compounds quantified for each category were the following: For PCBs, PCB 18, PCB 54, PCB 28, PCB 52, PCB 155, PCB 101, PCB 77, PCB 123, PCB 118, PCB 153, PCB 105, PCB 138, PCB 126, PCB 185, PCB 156, PCB 157, PCB 180, PCB 198, PCB 169, PCB 170, PCB 194, and PCB 209; for organochlorine pesticides, α-hexachlorocyclohexane (HCH), hexachlorobenzene, γ-HCH, *p,p′*-1,1-bis(4-chlorophenyl)-2,2-dichloroethylene) (DDE), *o,p′*-DDE, *p,p′*-1,1-dichloro-2,2,-bis(4-chlorophenyl)ethane) (DDD), *o,p′*-DDD, *p,p′*-DDT, and *o,p′*-DDT; for PAHs, naphthalene, 2-methylnaphthalene, 1-methylnaphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benz[*k*]fluoranthene, benz[*a*]pyrene, dibenz[*a,h*]anthracene, and benz[*ghi*]perylene.

Organotin compounds

Data regarding organotin contamination for the first three stations were derived from Pellizzato et al. [22]. The method for analysis of organotin compounds (OTCs) in stations 4 and 5 was the same as that reported in the previous paper [22]. The following compounds were analyzed: TBT, dibutyltin, monobutyltin, TPhT, diphenyltin, and monophenyltin.

Chemometric analysis

Concentrations of PCBs, organochlorine pesticides, and PAHs, as well as of OTCs in the same samples, were used to develop a chemometric model.

The partial least squares (PLS) regression in latent variables method was applied to search for a relationship between the analytical data and to build a model capable of explaining the extent of the phenomenon of imposex (i.e., the female penis length of *H. trunculus*) with the concentrations of pollutants.

The PLS analysis is a regression method based on principal component–like latent variables and is used for constructing predictive models. It allows extraction of principal components from original independent variables (predictor variables) and dependent variables (response variables) [25].

The PLS model was built starting from a matrix with 22 observations and five variables, four of which were independent (x block) and one of which was dependent (y). Initially, principal component analysis (PCA) was performed to observe the structure of the data. Based on the distribution of objects in the space defined by the principal components, the entire set of data was split into two similarly composed sets, called training and validation, which were used to build the model and to validate the model, respectively. To test the model's predictive ability and stability, the procedure was repeated using the validation set for training and the training set for validation. Finally, a PLS model with all data (training plus validation sets) was made to apply the largest number of available data in the model.

Parameters useful for interpreting model performance were r^2x and r^2y, which indicate the unitary variance of x and y variables, respectively, explained by each calculated principal component. The term q^2 is the variance explained in prediction, calculated by a random method. Standard deviation of error of calculation (SDEC) and standard deviation of error of prediction (SDEP) are the errors obtained from calculation of the y values of objects used to build the model and from calculation of the y values of objects belonging to the validation set, respectively; SDEC and SDEP were computed as follows:

$$\text{SDEC} = \left(\frac{\text{RSS}}{n}\right)^{1/2} \tag{1}$$

$$\text{SDEP} = \left(\frac{\text{PRESS}}{n}\right)^{1/2} \quad \text{where} \tag{2}$$

$$\text{RSS} = \sum_i (y_{i_{\text{observed}}} - y_{i_{\text{calculated}}})^2 \tag{3}$$

is the residual of sum of squares and

Table 1. Biological data of populations of *Hexaplex trunculus* sampled in the Lagoon of Venice, Italy[a]

Sampling stations	No. of organisms	M/F	Average female penis length (mm)	RPSI	VDSI
S. Nicolò del Lido	30	4/26	12 ± 2	36.2	4.9 ± 0.3
S. Maria del Mare	51	23/28	8 ± 2	8.03	4.1 ± 0.6
Chioggia	37	15/22	5 ± 3	1.03	3.6 ± 0.5
Lido Mare	48	28/20	9 ± 4	9.1	4.5 ± 0.3
Alberoni	60	21/39	6 ± 3	4.3	4.3 ± 0.2

[a] Modified from Pellizzato et al. [22]. M/F = male/female; RPSI = relative penis size index; VDSI = vas deferens sequence index.

$$\text{PRESS} = \sum_i (y_{i_{\text{observed}}} - y_{i_{\text{predicted}}})^2 \quad (4)$$

is the predictive error sum of squares.

Both SDEC and SDEP are to be compared with the range of the *y* variable.

Loadings are weights of the starting variables in the linear combination of principal components; the coefficients in the model equation express the contribution of each *x* variable to the total calculation of the *y* value.

The SIMCA-P software (Soft Independent Models of Class Analogy, Ver 8.0; Umetrics AB, Umeå, Sweden) was used for all chemometric processing. This software automatically autoscales data so that they have means of zero and a variance of one.

RESULTS

Table 1 lists the biological data of the samples from the five stations. The number of sampled organisms, number of males and females, mean of female penis lengths, RPSI, and VDSI are all reported. From biological data, it can be observed that whereas the VDSI is not able to discriminate the stations, the RPSI can differentiate them; the highest RPSI value was found in S. Nicolò del Lido and the lowest in Chioggia.

Table 2 lists PCB, organochlorine pesticide, and PAH concentrations in the visceral coil and in the rest of the soft body of the organisms. The sex of the organisms in every pool, the degree of imposex (expressed as VDS), and the average length of the penis also are listed. The data show that in all stations, the concentrations of all classes of pollutants were always higher in the visceral coil than in the rest of the soft tissues.

The percentage load of the pollutants for every station is shown in Figure 2. The load of a specific compound in a specific tissue is defined as the percentage ratio between the amount (ng) of pollutant in the visceral coil or in the rest of the soft body and the amount in the entire organism. Preferential accumulation (>80%) of PCBs and pesticides in the visceral coil rather than in the rest of the soft body was observed at every station; this result was statistically significant (*t* test, $p < 0.05$). The situation changes when PAHs are considered: Preferential bioaccumulation in one of the two parts of the gastropods was not observed (*t* test, $p > 0.05$) except at Lido Mare, where a higher accumulation of PAHs in the visceral coil was noted (Fig. 2c).

The average concentrations in the entire *H. trunculus* organism for every station are shown in Figure 3 as box-and-whisker plots. Considering PCBs (Fig. 3a), the most polluted specimens were collected from S. Nicolò del Lido, with concentrations in the entire organism ranging from 118 ± 4 (mean ± SD) to 363 ± 55 ng/g dry weight. The PCB level decreased in snails at S. Maria del Mare (from 45 ± 2 to 160 ± 30 ng/g dry wt), whereas at Lido Mare, concentrations ranged between 80 ± 6 and 131 ± 7 ng/g dry weight. Gastropods from Alberoni and Chioggia had the lowest levels of PCBs, with ranges of 34 ± 1 to 51 ± 1 ng/g dry weight and 13 ± 2 to 25 ± 2 ng/g dry weight, respectively. Considering pesticide concentrations (Fig. 3b) in the entire organism, mollusks at S. Nicolò del Lido (13 ± 1 to 51 ± 1 ng/g dry wt) again turned out to be the most polluted, followed by those from Lido Mare (19.2 ± 0.5 to 29 ± 2 ng/g dry wt). In samples from the other three stations, concentrations were always lower than 11 ng/g dry weight. Regarding PAHs, organisms collected at Alberoni (18–27 ng/g dry wt) were the least polluted, whereas the most polluted were snails from Lido Mare (59–123 ng/g dry wt) (Fig. 3c).

The congener compositions for each class of pollutants were as follows: Among PCBs, the most abundant congeners were PCB 153 and PCB 138, ranging from 33 to 46% and from 22 to 25%, respectively. Furthermore, PCB 118, PCB 101, and PCB 180 had abundances greater than 5% in snails from all stations. The number of congeners with concentrations exceeding the limits of detection was higher in organisms from S. Nicolò del Lido, S. Maria del Mare, and Lido Mare compared to those from the other two sites on the seaward side, Alberoni and Chioggia.

Among pesticides, the compound with the highest abundance was a product of DDT degradation, *p,p′*-DDE, which amounted to between 79 and 98% of the total sum of pesticides in the gastropods of all stations. Homogeneous distribution of congeners was not found for PAHs. Phenanthrene was one of the most abundant congeners, ranging between 17 and 26% in the mollusks of all stations.

A relationship was sought between PCB, organochlorine pesticide, and PAH concentrations, together with OTCs, and one of the anatomic modifications (i.e., penis length) induced in *H. trunculus* females affected by imposex. In Table 3, OTC concentrations and average penis length of each pool of organisms are reported. The correlation matrix yielded statistically significant correlation values ($p < 0.05$) between penis length and PCBs ($r = 0.74$), OTCs ($r = 0.70$), and organochlorine pesticides ($r = 0.64$). This stimulated more detailed chemometric analysis.

The matrix for chemometric processing was composed of the concentrations in the entire organisms of the four classes of pollutants analyzed (*x* block) and of the female penis lengths (*y* value), for a total number of 22 observations. The PCA was applied to the data set, confirming the different levels of contamination in the sampling stations as reflected by concentrations in the gastropods. Based on the PCA score plot picking samples from all groups identifying the stations, samples were divided into two sets, one for training and one for validation of the PLS chemometric model. A first PLS model, built with

Table 2. Sex, vas deferens sequence (VDS), average penis length, and concentrations (ng/g dry wt) of pollutants in pooled visceral coil and rest of soft body of *Hexaplex trunculus*[a]

Sampling stations (Lagoon of Venice, Italy)	Sex	VDS	Penis length (mm)	Visceral coil			Rest of soft body		
				ΣPCBs	ΣPesticides	ΣPAHs	ΣPCBs	ΣPesticides	ΣPAHs
S. Nicolò del Lido	M	—	17 ± 5	899 ± 20	80 ± 2	197 ± 9	—	—	—
	F	4	11 ± 2	1074 ± 161	36 ± 5	106 ± 16	34 ± 6	2.1 ± 0.3	55 ± 13
	F	4.7–5	12 ± 2	352 ± 8	69 ± 4	131 ± 56	26 ± 2	2.35 ± 0.03	39 ± 1
	F	5s	13 ± 1	654 ± 68	114 ± 9	71 ± 11	25 ± 2	2.4 ± 0.1	39 ± 13
	F	5s	13.1 ± 0.2	740 ± 33	91 ± 11	376 ± 41	—	—	—
	F	5s	10 ± 1	621 ± 25	140 ± 4	70 ± 8	63 ± 2	2.5 ± 0.1	45 ± 14
S. Maria del Mare	M	—	20 ± 2	403 ± 12	17.1 ± 0.2	179 ± 83	30 ± 1	1.268 ± 0.005	35 ± 1
	M	—	19 ± 3	453 ± 2	24 ± 3	38 ± 12	29 ± 5	1.33 ± 0.01	33 ± 8
	F	3	6 ± 2	329 ± 9	16 ± 4	82 ± 39	28 ± 8	3 ± 2	23 ± 3
	F	4–4.3	9 ± 2	503 ± 75	13 ± 2	46 ± 7	29 ± 2	1.35 ± 0.05	35 ± 3
	F	4–4.3	8 ± 2	109 ± 3	14 ± 3	39 ± 7	9 ± 2	1.6 ± 0.6	71 ± 32
	F	4–4.3	9 ± 1	549 ± 82	20.5 ± 0.2	128 ± 19	34 ± 14	1.6 ± 0.2	39 ± 5
	F	4.7–5	9 ± 3	333 ± 3	18.9 ± 0.5	69 ± 28	—	—	—
	F	4.7–5	10 ± 2	288 ± 43	18 ± 3	40 ± 6	21 ± 3	1.9 ± 0.3	29 ± 4
Chioggia	M	—	24 ± 2	62 ± 1	15.1 ± 0.3	94.5 ± 0.5	5.3 ± 0.3	1.3 ± 0.2	36 ± 2
	M	—	26 ± 7	42 ± 7	6.5 ± 0.8	107 ± 3	6 ± 1	1.06 ± 0.05	30.7 ± 0.4
	F	3	4.3 ± 0.5	33 ± 5	4.9 ± 0.7	83 ± 13	5.8 ± 0.8	1.3 ± 0.2	56 ± 5
	F	3	7 ± 5	73 ± 5	19.55 ± 0.05	91 ± 1	4.2 ± 0.4	1.37 ± 0.01	41.3 ± 0.1
	F	4	5 ± 1	45 ± 5	8.17 ± 0.09	89 ± 5	4.2 ± 0.3	0.774 ± 0.009	33 ± 2
	F	4	5 ± 3	48 ± 7	14 ± 2	110 ± 17	5.12 ± 0.02	1.44 ± 0.08	49 ± 1
	F	4–4.3	6 ± 2	54 ± 2	10 ± 1	101 ± 4	5.2 ± 0.3	0.86 ± 0.02	38.1 ± 0.8
	F	4–4.3	5 ± 2	48 ± 1	14.4 ± 0.9	130 ± 12	3.8 ± 0.3	4 ± 3	39 ± 1
Lido Mare	F	4	8 ± 1	129 ± 9	39.4 ± 0.5	172 ± 47	9 ± 1	1.5 ± 0.2	49 ± 7
	F	4.3	7 ± 3	151 ± 3	35.9 ± 0.9	94 ± 14	7.7 ± 0.2	1.66 ± 0.06	38 ± 3
	F	4.7	11 ± 3	169 ± 8	39.9 ± 0.5	85.0 ± 0.6	8.7 ± 0.9	1.8 ± 0.1	33.5 ± 0.3
	F	5s	11 ± 5	244 ± 13	53 ± 3	114.3 ± 0.7	9 ± 1	1.9 ± 0.6	29.8 ± 0.8
Alberoni	M	—	18 ± 6	128 ± 5	39 ± 5	34 ± 5	11 ± 1	2.2 ± 0.6	22 ± 2
	F	4	5 ± 4	99 ± 5	22 ± 5	26 ± 12	6.9 ± 0.4	1.8 ± 0.1	29 ± 2
	F	4.3	6 ± 2	63 ± 3	17.9 ± 0.2	35 ± 14	6.6 ± 0.3	1.7 ± 0.2	15
	F	4.7	7 ± 1	97 ± 19	16 ± 3	20.0 ± 0.2	9 ± 2	1.8 ± 0.2	16 ± 1

[a] PCBs = polychlorinated biphenyls; PAHs = polycyclic aromatic hydrocarbons; 5s = female at imposex stage 5 with spit capsule gland.

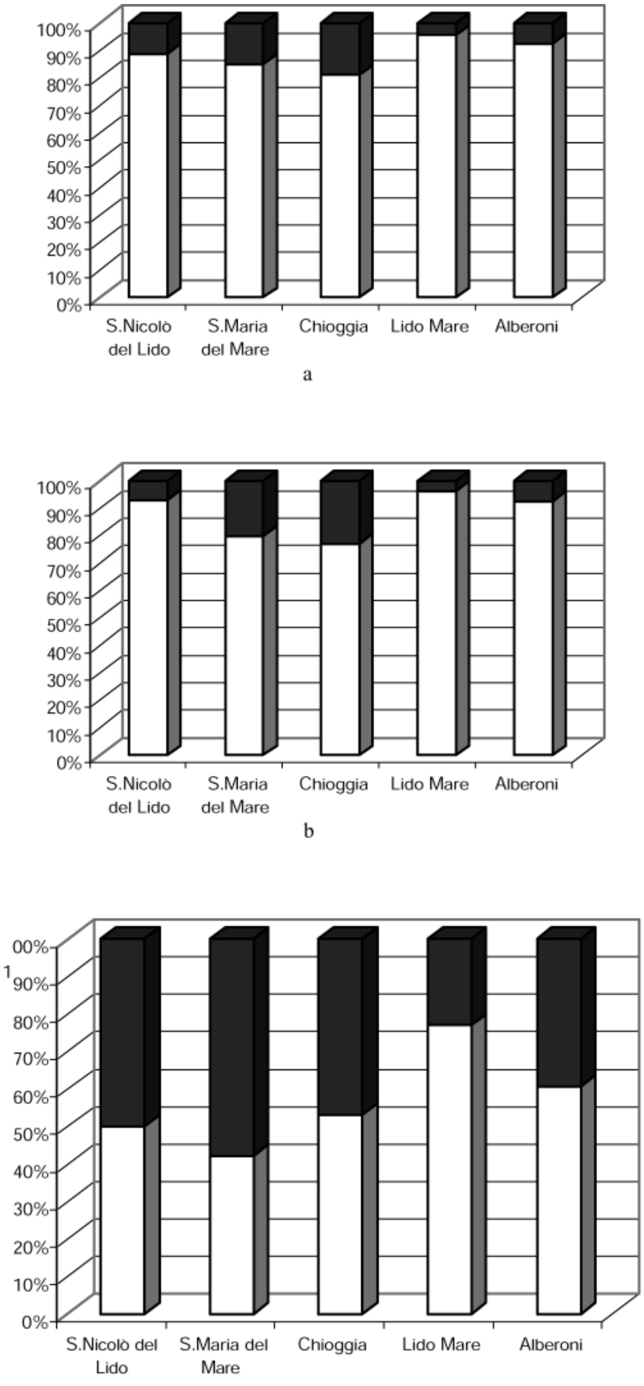

Fig. 2. Percentage loads of contaminants in visceral coil and in rest of soft body. (**a**) Polychlorinated biphenyls. (**b**) Organochlorine pesticides. (**c**) Polycyclic aromatic hydrocarbons.

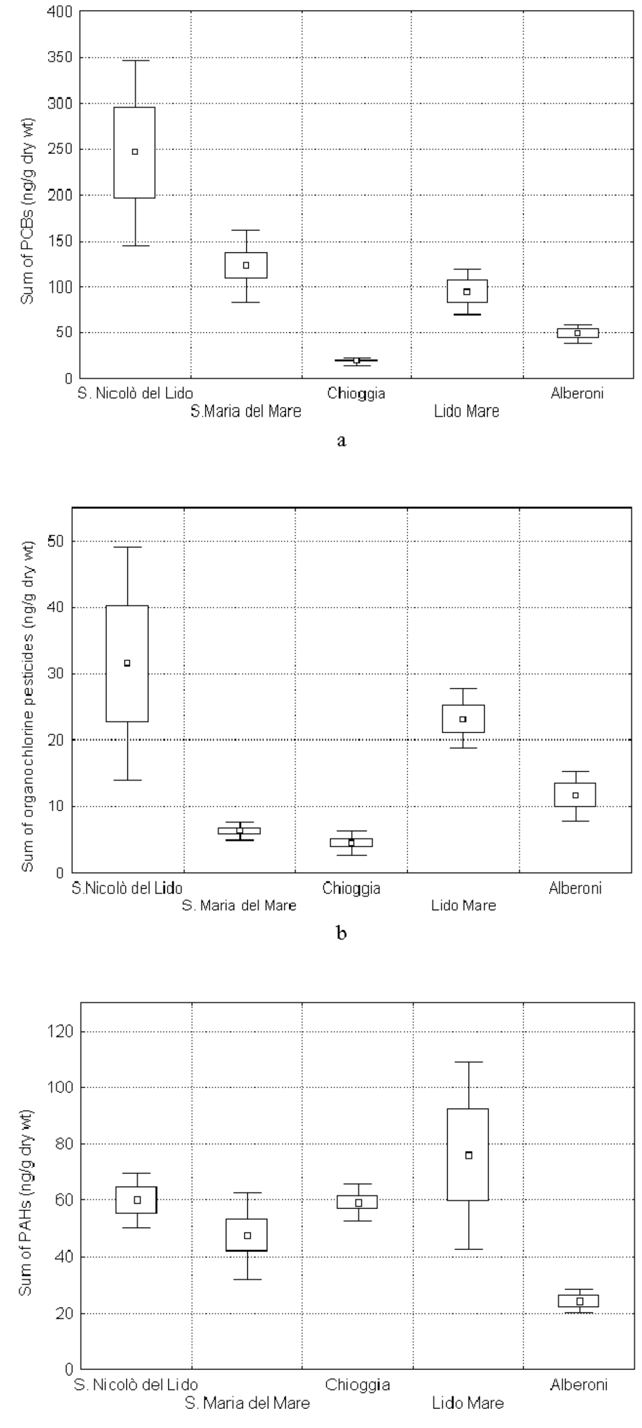

Fig. 3. Box-and-whisker plots of concentrations in entire organisms for each station (all stations located in Venice, Italy) of (**a**) Σpolychlorinated biphenyls (PCBs), (**b**) Σorganochlorine pesticides, and (**c**) Σpolycyclic aromatic hydrocarbons (PAHs).

Mean+Standard Deviation Mean+Standard Error
Mean−Standard Deviation Mean−Standard Error □ Mean

the training set and validated with the validation set, yielded the following equation:

$$\text{Penis length} = 2.72 + 0.31 \cdot \sum \text{PCBs} + 0.29 \cdot \sum \text{Pesticides} + 0.12 \cdot \sum \text{PAHs} + 0.28 \cdot \sum \text{OTCs} \quad (5)$$

The coefficients of the equation indicate that PCBs, organochlorine pesticides, and OTCs act in the same manner in modeling penis length, whereas the contribution of PAHs, although in the same direction as the other classes of compounds, was less important. The parameters obtained from the model ($r^2x = 0.63$, $r^2y = 0.70$, $q^2 = 0.52$, SDEC = 1.58, and SDEP = 2.34) show that the model is able to explain between 63 and 70% of data variance in fitting and 52% in prediction. Errors are acceptable when compared with the variation range of the *y* variable (penis length; 4–13 mm).

Table 3. Organotin compound (OTC) concentrations and penis length of each pool

Name of observation[a]	ΣOTCs (ng Sn/g dry wt)	Penis length (mm)
4N	230	11
4.7–5N	277	12
5s(1)N	403	13
5s(3)N	442	10
3S	151	6
4–4.3(1)S	155	9
4–4.3(2)S	147	8
4–4.3(3)S	212	9
4.7–5S	138	10
3(1)C	129	4.3
3(2)C	113	7
4(1)C	106	4.9
4(2)C	129	5
4–4.3(1)C	134	6
4–4.3(2)C	110	5
4L	219	8
4.3L	182	7
4.7L	224	11
5sL	243	11
4A	150	5
4.3A	242	6
4.7A	197	7

[a] The name of each observation is indicated as follows: Numbers indicate the imposex degree of the pools; numbers in brackets indicate that more than one pool with the same imposex degree are present in the station; letters indicate the sampling stations located in Venice, Italy (N = S. Nicolò del Lido; S = S. Maria del Mare; C = Chioggia; L = Lido Mare; A = Alberoni).

A second PLS model was built using the original validation set for training and the training set for validation, yielding the following equation:

$$\text{Penis length} = 3.40 + 0.33\cdot\sum \text{PCBs} + 0.26\cdot\sum \text{Pesticides} + 0.07\cdot\sum \text{PAHs} + 0.29\cdot\sum \text{OTCs} \quad (6)$$

The same information concerning the contributions of variables for the first model was obtained from the second model, and in this case, the model parameters also were similar ($r^2x = 0.63$, $r^2y = 0.66$, $q^2 = 0.51$, SDEC = 1.26, and SDEP = 2.13). The stability of the chemometric model was therefore ascertained.

Finally, a model using all the available data was built, and the following equation was obtained:

$$\text{Penis length} = 3.03 + 0.30\cdot\sum \text{PCBs} + 0.26\cdot\sum \text{Pesticides} + 0.06\cdot\sum \text{PAHs} + 0.28\cdot\sum \text{OTCs} \quad (7)$$

The role of organic compounds in explaining the imposex modification was confirmed, and the following fitting and prediction capacities of the model were produced: $r^2x = 0.63$, $r^2y = 0.59$, $q^2 = 0.45$, and SDEC = 1.64.

It should be noted that with all three models, the obtained values of SDEC and SDEP, when compared with the range of the *y* variable, indicate that errors in fitting and in prediction, respectively, are acceptable.

Table 4 lists the loadings of the PLS component for the three models. As evidence of the stability of the model, the loadings were within a range of variation of 5% for PCBs, pesticides, OTCs, and penis length, and only for PAHs, the least important variable, was the range of variation within 30%.

DISCUSSION

Uptake of contaminants and their partitioning in tissues of H. trunculus

The principal mechanisms of contaminant uptake by aquatic organisms are either direct contact between the compound and the organism body or respiratory surfaces or ingestion of contaminated food. In particular, organochlorine compounds probably adsorb on the external surfaces of marine organisms, partition into the hemolymph or blood plasma lipoproteins, and are transported to lipid-rich tissues [26]. The higher lipid content in the visceral coil than in the rest of the soft body thus explains the difference in the bioaccumulation between these two parts of the organism found in the present work. In addition, given the feeding behavior of *H. trunculus*, an important way for assimilation of pollutants by this species probably is trophic, and the visceral coil represents the main part of the organism that comes into contact with the pollutants contained in food.

Notwithstanding the different weights of the visceral coil and the rest of the soft body, which usually are greater for the latter, PCBs and organochlorine pesticides show higher loads in the former. Being carnivorous, *H. trunculus* accumulates and magnifies the concentrations of contaminants contained in the organisms on which it feeds. It may be hypothesized that the detoxification system of the visceral coil cannot break down these organic compounds: They are transferred to the rest of the soft body more slowly than their introduction into the organism, and the greatest load therefore is found in the visceral coil. Polycyclic aromatic hydrocarbons also can bioaccumulate in organisms, but as a result of metabolism and excretion, accumulation in the food chain is not observed [7]. It may be suggested that *H. trunculus* is able to break down PAHs better than PCBs and organochlorine pesticides. The result of degradation of PAHs and their contemporaneous transfer from the visceral coil to the rest of the soft body is nonpreferential bioaccumulation in one of the two parts.

Sources of contamination in H. trunculus

With reference to PCBs and organochlorine pesticides, a clear distinction was found among the levels of concentration in the gastropods from the stations, especially S. Nicolò del Lido and the stations at sea. These differences may be explained by considering the different sources of pollution inside the lagoon. For example, at S. Nicolò del Lido, organisms are

Table 4. Loadings of the first partial least square component or three chemometric models[a]

	ΣPCBs	ΣPesticides	ΣPAHs	ΣOTCs	Penis length
Model 1	0.59	0.56	0.22	0.54	0.53
Model 2	0.64	0.50	0.14	0.56	0.51
Model 3	0.61	0.53	0.12	0.58	0.49

[a] PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls; OTCs = organotin compounds.

exposed to high PCB and pesticide concentrations because of the release of discharge water from the industrial area of Porto Marghera and runoff from the agricultural areas bordering the lagoon.

Although S. Maria del Mare is situated close to the Malamocco—Marghera Canal, which connects Malamocco inlet with the industrial area of Porto Marghera, it is a relatively clean station considering the organic compounds studied: Low PCB and pesticide levels were detected in the gastropods collected there. According to other authors [27], this area is, in effect, characterized by a negligible anthropogenic impact, shallow waters, occurrence of sea grass beds, and high tidal exchange. Several algal species tolerating low pollution levels were sampled at this station. The contamination level in organisms from Chioggia was very low, as expected from a reference site, and was influenced by dilution resulting from the position of the station in the open sea.

Instead, no significant differences among snails from the various stations were found for PAHs, except at Lido Mare. Contamination by this class of compounds is quite common throughout the environment because of the nature of the compounds and the widespread processes by which they are produced (e.g., boat engine emissions).

The high average PAH concentration found in whole organisms at Lido Mare is explained by its position near the Lido inlet, connecting the Adriatic Sea with the lagoon. Through this inlet, commercial shipping and tourist pleasure craft enter the lagoon, and recent, continuous heavy inputs of PAHs probably occur. This is supported by the fact that higher loads of PAHs were found in the visceral coil compared to those in the rest of the soft body. Accumulations of contaminants resulting from the hydrodynamics of the North Adriatic also have been highlighted in this area [28].

Unexpectedly, in organisms from S. Nicolò del Lido, PAH concentrations were lower than those at Lido Mare and comparable with those at Chioggia and S. Maria del Mare, probably because of a combination of two effects. Environmental degradation is caused, on the one hand, by oxygenation resulting from water exchange with the sea during tidal cycles and, on the other, by the simultaneous metabolism of PAHs in the gastropods.

The distribution pattern of PCBs reflects the type of pollutant sources to which the organisms are exposed. The stations S. Nicolò del Lido, S. Maria del Mare, and Lido Mare are more influenced by the industrial area than the stations on the seaward side are, and organisms from these stations have a PCB pattern that is richer in congeners. In any case, the most common PCB congeners in tissues of *H. trunculus*, PCB 153 and PCB 138, are the most common in biota tissues as well [29]. These are hexachlorinated biphenyls, which are among the most resistant to degradation.

The most abundant pesticide congener in the gastropods of every station was a metabolite of DDT, *p,p′*-DDE, formed from DDT by dehydrochlorination. The rate of this degradation reaction probably relates to some general detoxification processes and increases from invertebrates to fish, birds, and mammals [30]. The congener *p,p′*-DDE is recognized as the major and persistent metabolite of DDT [6].

Relationship of PCBs, organochlorine pesticides, and PAHs with female penis length

To our knowledge, the role of PCBs, pesticides, and PAHs acting as endocrine disruptors in the promotion of imposex in *H. trunculus* has never been hypothesized, although different papers [19–21] have highlighted the possible role of compounds other than TBT in the promotion of imposex in several species of gastropods.

The endocrine system is clearly involved in imposex, whichever hypothesis is considered regarding the induction of the biochemical mechanism of imposex: The inhibition of the cytochrome P450–dependent aromatase responsible for the conversion of androgens to estrogens [11,12], inhibition of the excretion of testosterone [13], or release of a neuropeptide hormone (penis morphogenic factor), which normally induces male differentiation in mollusks [14]. It has been claimed that TBT is the main agent in these mechanisms, and it has been suggested that its action is mediated by androgen receptors (ARs) [12,31].

However, TBT is not the only compound able to interact with ARs. Experimental evidence has shown that PCBs, pesticides, and PAHs interact with ARs, but until now, the few studies of their interaction with AR have been conducted mostly in vitro or in vivo on rats or, more generally, on vertebrates [5,6,8,9,32–35]. Few studies on invertebrates and, especially, on gastropods have been published.

From the literature, it is known that PCBs can bind AR either to activate it or to inactivate it. In particular, di-*ortho*-substituted PCB 138, one of the most abundant PCB congeners found in tissues of *H. trunculus*, competes with the natural ligand for binding to AR [5]. Kelce et al. [6] have shown that *p,p′*-DDT, *p,p′*-DDD, and *p,p′*-DDE also are able to bind to the AR. Vinggaard et al. [33] observed that some PAHs, including benzo[*a*]anthracene, benzo[*a*]pyrene, fluoranthene, and chrysene, also act on AR. Therefore, the potential cannot be excluded that other compounds may act in the same way as TBT through AR in the induction of imposex. Some experimental studies in this direction should be undertaken. Nevertheless, it also is evident that further studies are needed to clarify the role of steroid hormone receptors in gastropods. Understanding analogies between AR in vertebrates and invertebrates would be useful when applying results obtained in the former to the latter.

Several endocrine disruptors are present simultaneously in the environment. It is very difficult to know how they behave in mixture, however, because of the complexity of the mechanisms involved, the potential effects that these contaminants can exert on different parts of the body, and the role that other factors (e.g., stage of development and differences between species, age, and season) may play in these effects [36].

One of the main findings of the present work is that *H. trunculus* is simultaneously exposed to various classes of potential endocrine disruptors in the Lagoon of Venice. Attempts to gain insights regarding the possible synergistic role of these environmental pollutants by means of a stable chemometric model indicate that PCBs and organochlorine pesticides probably have a potential influence on one of the anatomic modifications induced by imposex, together with the undoubted role played by TBT. As widely discussed by Pellizzato et al. [22], the presence of TBT and TPhT in the Lagoon of Venice is the result of marine paints applied on the hulls of ships and submerged harbor facilities to prevent development of fouling. In addition to antifouling paints, TPhT is used in agriculture and may enter the lagoon in freshwaters from the drainage basin. The TBT concentrations were found to be distributed homogeneously between visceral coil and the rest of the soft body, whereas its degradation products, dibutyltin and monobutyltin, were found to be higher in the visceral coil. In addition, TPhT was found almost exclusively in the visceral coil. However, it should not be excluded that other chemicals present in the environment but not yet known or analyzed also may play a role in the induction of this phenomenon.

Once more, few studies have examined the synergistic effects among endocrine disruptors as regards invertebrates, and they highlight the fact that this issue is still controversial. For example, in a study conducted on Japanese medaka, *Oryzias latipes* (Jordan and Snyder, 1906), the contemporaneous administration of TBT and a congener of PCBs showed an agonistic effect on egg maturation and fertilization processes but an antagonistic effect during embryological development [37]. A study on the female offspring of Wistar rats [38] showed that TBT has some androgenic action through an AR-mediated transcription mechanism, whereas DDE, an AR antagonist, prevents this action. This result is in contrast with the agonistic effect hypothesized in the present study between OTCs and organochlorine pesticides. Nevertheless, because differences between species in the functioning of the endocrine system are not sufficiently known, speculations regarding *H. trunculus*, on the basis of results obtained from widely different taxa, may be misleading.

CONCLUSION

The present work shows that organic pollutants, such as PCBs and organochlorine pesticides, although banned several years ago, are still present in the Lagoon of Venice and accumulate in organisms. Concentrations of PCBs, pesticides, and PAHs were studied in the visceral coil and other soft tissues of *H. trunculus*, and preferential accumulation of PCBs and pesticides was noted in the former. On average, PAHs were equally partitioned between the two tissues. The concentrations calculated in the entire organisms distinguished between the contamination level of sampling stations characterized by different sources of pollution and, especially, discriminated stations inside the lagoon from stations outside it.

Moreover, the present study examined the role of these organic pollutants and of OTCs in promoting one of the anatomic modifications induced in imposex. A stable chemometric analysis modeled the female penis length with the various classes of endocrine disruptors, indicating that a synergistic effect is possible: PCBs, organochlorine pesticides, and OTCs act in the same direction and with similar intensity, whereas the contribution of PAHs, although still positive, is much lower.

The authors consider these results, based on correlation analysis, as preliminary, and they recognize the need for more experimental work to ascertain the role of these contaminants in inducing endocrine disruptions and their possible synergic effects. Laboratory assays on the effects of poisoning mollusks with several endocrine disruptors could be worthwhile, because organisms in the environment are simultaneously exposed to several classes of contaminants. Nevertheless, interpretable experimental results can be achieved only if more basic research on invertebrate endocrinology (e.g., steroid hormone receptors of gastropods or comparative and ecological aspects of endocrine disruptors) is undertaken, as recently advocated by Oehlmann and Schulte-Oehlmann [39].

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