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Environmental risk assessment of Polish wastewater treatment plant activity

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h i g h l i g h t s

- Bioassays prove to be efficient tool in EIA.
- Water treatment greatly affects water bodies receiving WWTP effluents.
- Treatment of wastewaters transforms their matrix interactions.

a b s t r a c t

Wastewater treatment plants (WWTPs) play an extremely important role in shaping modern society's environmental well-being and awareness, however only well operated and supervised systems can be considered as environmentally sustainable. For this reason, an attempt was undertaken to assess the environmental burden posed by WWTPs in major Polish cities by collecting water samples prior to and just after wastewater release points. Both classical and biological methods (Microtox®, Ostracodtoxkit F™ and comet assay) were utilized to assess environmental impact of given WWTP. Interestingly, in some cases, water quality improvement indicated as a toxicity decrement toward one of the bio-indicating organisms makes water worse for others in the systems. This fact is particularly noticeable in case of Silesian cities where heavy industry and high population density is present. It proves that WWTP should undergo individual evaluation of pollutant removal efficiency and tuned to selectively remove pollutants of highest risk to surrounding regional ecosystems. Biotests again proved to be an extremely important tool to fully assess the impact of environmental stressors on water bodies receiving effluents from WWTPs.

Keywords: Environmental stressors removal, Ecotoxicity, Wastewater treatment plants, Chemometrics, Biotests

1. Introduction

Biotests are conducted to prove the presence of environmental stressors' mixture, show their combined effect and holistic impact on the environmental compartments (Kapanen et al., 2013; Kudłak et al., 2014, 2015; Manusadzianas et al., 2003; Pessala et al., 2004; Tigini et al., 2011; Tsakovski et al., 2009). For this reason, bioassays can be conducted on unicellular and microcosm systems where organisms from different trophic levels are sensitive to different toxins (Szczepańska et al., 2016; Dubiella-Jackowska et al., 2010).

Another advantage of biotests is the possibility of detecting what is of great importance in view of the possible carcinogenic properties of environmental stressors: the endocrine and mutagenic potential of tested samples. Current knowledge in this field proves that toxicity may be the result of:

- the interactions of toxins with receptors,
- the breaking down of the molecular membrane,
- chemical reactions with cell elements,
- the inhibition of enzymatic activity (Cohen and Van Heyningen, 1982; Kudłak et al., 2015).

Bioassays constitute an important branch of analytics and gain more and more interest next to classical instrumental methods in conducting environmental impact assessments (EIA) (Kokkali and

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Van Delft, 2014). Due to increasing consumption of pharmaceuticals and also industrial/agricultural chemicals the impact on water bodies increases. Simple instrumental determination of stressor concentration levels will never give a full answer on the real threat posed by a vast number of substances reaching WWTPs (Frenzilli et al., 2009; Ohe et al., 2004; Gana et al., 2008). In the present state, one common approach is to link chemical concentrations to toxicity data. This is a typical univariate strategy which relies on traditional Quality Guidelines.

For these reasons, an effort has been undertaken to determine the possibility of utilizing biotests to assess efficiency of pollutant removal in the industrial and municipal waste water treatment plants of Poland and to determine the burden placed upon water bodies receiving theoretically treated waste waters. The selected battery of tests has been to respond to both acute and chronic toxicity at cellular and higher levels of biota organization.

2. Methodology

2.1. Instruments, chemicals and reagents

Chemicals used for Microtox® and Ostracodtoxkit F™ were purchased from ModernWater Ltd. and MicroBiotests, Inc., respectively. These included 2% NaCl solution, lyophilized *Vibrio fischeri*, Microtox Diulent, Microtox Acute Reagent, Osmotic Adjusting Solution, Reconstitution Solution, vials with algal food for chronic toxicity tests and matrix dissolving medium, spiruline, 6-well test plates, and certified dormant eggs of *Heterocypris incongruens*. Epithelial colon cancer cells HT-29 were obtained from American Type Culture Collection (Manassas, USA). McCoy's 5a (Modified) Medium, supplemented with 10% foetal bovine serum and antibiotic (1% penicillin-streptomycin), DMSO (CAS no. 67-68-5), H_2O_2 (CAS no. 21-67-63), N_2EDTA (CAS no. 6381-92-6), trypsin-EDTA solution, NaCl (CAS no. 7647-14-5), NaOH (CAS no. 1310-73-2), Trizma®-base (CAS no. 77-86-1), Trizma® hydrochloride (CAS no.1185-53-1), phosphate buffered saline (PBS), Triton™ X-100 (CAS no. 92046-34-9), low and normal melting points agarose, trypsin-EDTA and SYBR® GREEN I nucleic acid gel stain were purchased from Sigma-Aldrich (Germany). Sterile serological pipette (25, 10 and 5 ml), cell cultures bottles, UltraFine™ tips, coverslips, microscope slides, 10-ml syringes, sterile centrifuge tubes and filters were purchased from VWR (Poland). All reagents were of analytical grade purity or better, in the case of reagents for microbiological purposes. The instruments and equipment used during the study were: Microtox® 500 of Modern Water Ltd., electronic pipettes (Rainin, Eppendorf), analytical balance from Radwag (Poland), CP411 Metron pH-meter (Poland), and a binocular microscope from Ceti NV (Belgium).

2.2. . Sampling

Sewage water samples were collected in 2012–2013 from 76 WWTPs receiving effluents from major Polish cities, each time at 2 points for every WWTP: in the hydrologic course prior to inflow of wastes (PO), and from the water course after release of wastes from the WWTP (ZO). Water samples were collected in the largest Polish cities. Data on technological processes taking place in particular WWTPs were collected from annual reports of Voivodship Environmental Protection Inspectorates (Poland). Water samples were collected in glass bottles and stored at 4 °C prior to being transported to a laboratory, filtered with a Cronus 25 mm PES Sterile Syringe Filter (0.2 μm) and frozen.

2.3. Instrumental

Major ions (Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, F^-, Cl^-, Br^-, NO_2^-, NO_3^-, SO_4^{2-}, PO_4^{3-}) were determined with a Dionex 3000i chromatograph (column: Ion Pac®AS22 (2 × 250 mm)); injection volume: 5 μL; suppressor: ASRS-300, 2 mm, mobile phase: 4.5 mM CO_3^{2-}, 1.4 mM HCO_3^-, flow rate: 0.38 ml/min, detection: conductivity, column: Ion Pac® CS14 (3 × 250 mm); suppressor: CSRS-300, 2 mm, mobile phase: 38 mM metasulfonic acid, flow rate: 0.36 ml/min, detection: conductivity (DIONEX, USA). Total organic carbon was measured with Shimadzu TOC-V CSH analyser. Metals (Na, K, Ca, Mg, Cu, Cd, Ni, Zn, Cr, Co, Fe) were determined with SensAA (GBC, Poland). All instrumental analyses were conducted with standard calibration curve methods.

2.4. Biotests

2.4.1. Microtox®

The Microtox® biotest utilizes *Vibrio fischeri* bacteria and their ability to bioluminescence. Acute toxicity was assessed by determining inhibition of the luminescence of the marine Gram-negative bacterium *Vibrio fischeri* (Leusch et al., 2014; Weltens et al., 2014), after a 30-min exposure to different samples. The bacteria were purchased in freeze-dried form and activated by rehydration with a reconstitution solution (specially prepared nontoxic Ultra-Pure Water) to provide a ready to-use suspension of organisms. The light emission of this bacterium in contact with different samples and exposure times was measured using the Microtox 500 analyser and bioluminescence inhibition was calculated and utilized as an endpoint for chemometric studies. The data were processed using the Microtox Omni Software, according to the Basic Test Protocol (81.9%). The design of the procedure is presented in Supplementary Figure A. Chromium sulphate was used as a positive control of the test.

2.4.2. Ostracodtoxkit F™

Ostracodtoxkit F™ is the best known and first biotest for direct contact of crustaceans with freshwater and brackish samples. Unlike bacteria, ostracods have a fully developed gastrointestinal tract, through which toxic substances can enter an organism (easily bioavailable pollutants can also enter via body shells and gills). An Ostracodtoxkit F™ toxkit containing vials with *Heterocypris incongruens* cysts, vials with spiruline and algae, reference sediment and dissolving medium were purchased from MicroBioTests, Inc. (Belgium). An optical microscope was used to assess the number of living organisms and for measurements of the length of the organisms according to the procedure presented in Supplementary Figure B. (Kudłak et al., 2011). Growth inhibition and mortality (according to the producer's and ISO 14371:2012 guidelines) were considered as endpoints for chemometric studies. Control organism growth of 400 μm and mortality of <20% are considered positive indicators for a test.

2.4.3. Comet assay

Epithelial colon cancer cells (HT-29, obtained from American Type Culture Collection, Manassas, USA) were grown in a monolayer culture at 37 °C in a humidified atmosphere of 5% CO_2 in McCoy's 5a (Modified) Medium, supplemented with 10% foetal bovine serum and antibiotic (1% penicillin-streptomycin) in a culture flask. The medium was changed twice a week. Single cell suspensions were prepared with a trypsin-EDTA solution (diluted 10 times) and finally re-suspended in McCoy's 5a (Modified) Medium, supplemented with serum and antibiotics.

2.5. Sample preparation for genotoxic investigations

To avoid hypo-osmotic shock, which may cause cytotoxic effects resulting in a false genotoxic effect later on (Lah et al., 2005), 5 ml of 10 × concentrated PBS was added into 45 mL of the sample. As a positive control, H_2O_2 was used. The samples were sterilized through a Cronus 25 mm PES Sterile Syringe Filter (0.2 μm) (Durgo et al., 2009; Kazimirova et al., 2012; Mihaljevic et al., 2011).

2.6. Alkaline comet assay

The comet assay was performed under alkaline conditions (Fairbairn et al., 1995), as described by Kazimirova et al. (2012) with some modifications. The cells were mixed with 0.5% low melting point (LMP) agarose in PBS at 37 °C and placed on glass microscope slides pre-coated with 1% normal melting point (NMP) agarose (2×10^4 cells/gel). Gels were covered with a cover slip and kept on ice for 5 min. Two gels per glass slide were prepared. For the purpose of this study, protocol for the exposure of slides, which was previously described by Lah et al. (2005) and later developed by Durgo et al. (2009) and Mihaljevic et al. (2011) was adopted. Slides were immersed into a water sample for 30 min at 37 °C. As a positive control, cells were exposed to 200 μM H_2O_2 in PBS for 20 min at 4 °C. Following the treatment of cells *ex vivo,* slides were washed with PBS and then subjected to lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Trizma®-base, pH 10 and 10% Triton X-100). After lysis, slides were placed in a horizontal electrophoresis tank and DNA was allowed to unwind for 20 min in electrophoretic buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13.3, 4 °C) before electrophoresis was performed for 20 min at 26 V (300 mA). After neutralization by washing gels three times, each for 5 min in 0.4 M Trizma®-base (pH 7.5) at 4° C, and then in water and 70% EtOH, slides were dried at room temperature. After staining the slides with SYBR GREEN I solution, the comets were detected and quantified as described below.

For quantitative analysis of nuclear DNA damage, the slides were viewed at 50 × magnification with an epifluorescence microscope Zeiss Imager Z2. Microscopic images of comets were captured by digital camera (CoolCube1) connected to a computer, and the comets were scored using Metapher 4 Computer Software. To test for significant differences between groups, the Kolmogorov-Smirnov test was used (Mouchet et al., 2006). The data were expressed as the mean values with the standard deviations (means ± SD) of the three independent experiments and were used as numeric values for chemometric studies.

2.7. Data treatment

Cluster analysis (CA) is a well-known and widely used data mining approach for various purposes, with hierarchical and non-hierarchical algorithms (Massart and Kaufman, 1983). To cluster objects characterized by a set of variables (e.g., sampling sites by chemical concentrations), one has to determine their similarity. A preliminary step of data standardization is necessary (autoscaling or z-transform) where normalized dimensionless numbers replace the raw data values. Thus, even serious differences in absolute values in the data set are reduced to close numbers. Then, the distance (being a measure of similarity) between the objects in the variable space has to be calculated. Very often the Euclidean distance is used for as similarity measure. Another way of measuring similarity is calculation of the correlation coefficient between the objects. Thus, from the input matrix, a similarity matrix could be constructed. There is a wide variety of hierarchical algorithms for object linkage, but the typical ones include single linkage, complete linkage, average linkage methods and Ward's method. The representation of the results of the cluster analysis is usually performed by a tree-like scheme called a dendrogram comprising a hierarchical structure (large groups are divided into small ones). The hierarchical methods of clustering mentioned above are agglomerative.

Principal components analysis (PCA) is a typical display method, which allows for the estimation of internal relations in the data set. There are different variants of PCA, but basically, their common feature is that they produce linear combinations of the original columns in the data matrix responsible for the description of the variables characterizing the objects of observation. These linear combinations represent a type of abstract measurements (factors, principal components) being better descriptors of the data structure than the original measurements. Usually, the new abstract variables are called latent factors and receive conditional names, depending on latent factor role in data interpretation. It is a common finding that just a few of the latent variables account for a large part of the data set variation. Thus, the data structure in a reduced space can be observed and studied (Massart et al., 1998).

Generally, when analysing a data set consisting of n objects for which m variables have been measured, PCA can extract f principal components, PCs, (factors or latent variables) where f < m. The first PC represents the direction in the data containing the largest variation. PC 2 is orthogonal to PC 1 and represents the direction of the largest residual variation around PC 1. PC 3 is orthogonal to the first two and represents the direction of the highest residual variation. The projections of the data on the plane of PC 1 and PC 2 can be computed and shown as a plot (score plot). In such a plot it is possible to distinguish similar groups. The PCs are a weighted sum of the original variables. The weights of the original variables are called loadings and give information about principal component origin.

3. Results and discussion

The basic statistics of the input data set is presented in Table 1. The measurements below the respective heavy metal limit of detection (LOD) were replaced by half of detection limit value – LOD/2.

Principal components analysis (PCA) and cluster analysis (CA)

Table 1
Basic statistics of the data set (n = 126).

	Units	Mean	Median	Minimum	Maximum	Std. Dev.
pH	–	7.15	7.11	4.93	8.14	0.462
COND	μS/cm	0.632	0.533	0.230	1.988	0.3639
TOC	mg C/L	41.72	37.02	5.810	119.1	21.24
Na^+	mg/l	27.17	11.26	0.250	222.8	42.34
K^+	mg/l	5.41	2.66	0.01	43.9	7.24
NH_4^+	mg/l	1.52	0.655	0.007	49.97	4.876
Mg^{2+}	mg/l	8.40	5.60	0.32	44.41	9.25
Ca^{2+}	mg/l	39.16	31.87	0.220	142.97	23.09
F^-	mg/l	0.45	0.032	0.032	30.58	2.74
Cl^-	mg/l	56.04	24.68	1.04	418.8	84.99
NO_2^-	mg/l	0.854	0.335	0.022	7.56	1.22
Br^-	mg/l	0.56	0.032	0.012	4.93	0.89
NO_3^-	mg/l	5.99	3.47	0.009	162.6	14.83
PO_4^{3-}	mg/l	0.682	0.032	0.008	21.10	2.224
SO_4^{2-}	mg/l	50.96	37.40	1.98	360.9	48.77
BLINH	%	−20.59	−25.50	−91.00	99.00	35.18
MORT	%	10.16	6.00	0.00001	53.00	10.33
GRINH	%	5.69	6.00	−45.00	73.00	18.75
Cd	mg/l	0.021	0.0015	0.0005	0.195	0.0401
Cr	mg/l	0.038	0.0005	0.0005	0.213	0.0541
Co	mg/l	0.068	0.056	0.0005	0.289	0.0641
Fe	mg/l	0.745	0.729	0.646	0.868	0.0565
%DNA	%	19.12	15.37	5.31	50.95	11.04

were utilized to find hidden relationships between pollutants and ecotoxicological results. It has to be stated that in the data set, some sites are lacking, or some PO and ZO indications are united. The interpretation and calculations are performed only on the complete couples (both PO and ZO indications) to make some classifications of spatial (geographical) distribution of the WWTPs.

3.1. Results of CA

CA results show the spatial grouping of WWTPs and could deliver preliminary information about pollution level of each identified group. As seen in Fig. 1, three major clusters are formed:

K1: 56, 55, 54, 57, 45, 62, 52, 60, 61, 70, 58, 48, 50, 40, 39, 25, 35 – all 17 objects with PO and ZO; 53, 43, 68, 66, 65, 63 (only PO indication) and 71, 72, 37 (only ZO indication) (Tomaszów Mazowiecki, Tychy, Prudnik, Nysa, Maszewo, Siedlce, Biała Podlaska, Lublin, Zamość, Kraśnik, Rzeszów, Przemyśl, Sanok, Nowy Sącz, Nowy Targ, Oświęcim, Stalowa Wola);

K2: 23, 30, 26, 27, 49, 15, 17, 20, 16, 19, 21, 14, 18, 13, 12, 8, 7, 4, 2, 3, 6 – all 21 (objects with PO and ZO indication); 11, 9, 24, 31, 46, 59 (only PO indication): 22, 53, 51, 65, 28, 10, 1 (only ZO indication) (Malbork, Starogard Gdański, Bartoszyce, Giżycko, Suwałki, Nowa Wieś Ełcka, Łapy, Łomża, Słupsk, Kołobrzeg, Szczecin, Police, Stargard Szczeciński, Gorzów Wielkopolski, Piła, Wałcz, Łowicz, Piotrków Trybunalski, Bełchatów, Częstochowa, Łuków);

K3: 69, 42, 41, 34, 64, 74, 73, 32 (all 8 objects with PO and ZO indication); 71, 72, 37, 1, 51, 28, 36 (only with PO indication); 43, 68, 66, 63, 9, 31, 11, 46, 24 (only ZO indication) (Katowice, Chorzów, Opole, Brzeg, Kraków Balice, Sandomierz, Zielona Góra, Żory).

Three patterns of pollution levels of WWTPs were conditionally named "low", "intermediate" and "high" with respect to the averages for all chemical and ecotoxicity parameters (Table 2).

As seen, there are only two maximal averages (Cr, BLINH) in K1; therefore, this cluster reflects grouping samples with "low" pollution (Tomaszów Mazowiecki, Tychy, Prudnik, Nysa, Maszewo, Siedlce, Biała Podlaska, Lublin, Zamość, Kraśnik, Rzeszów, Przemyśl, Sanok, Nowy Sącz, Nowy Targ, Oświęcim, Stalowa Wola). Grouped samples in K2 have generally "intermediate" levels of pollution, but with significant organic impact and hence, a high ecotoxicity response (Malbork, Starogard Gdański, Bartoszyce, Giżycko, Suwałki, Nowa Wieś Ełcka, Łapy, Łomża, Słupsk, Kołobrzeg, Szczecin, Police, Stargard Szczeciński, Gorzów Wielkopolski, Piła, Wałcz, Łowicz, Piotrków Trybunalski, Bełchatów, Częstochowa, Łuków). K3 reflects locations with "high" levels of pollution with significant inorganic impact (Katowice, Chorzów, Opole, Brzeg, Kraków Balice, Sandomierz, Zielona Góra, Żory).

Table 2
Cluster average values (highest values are marked with bold).

	K1	K2	K3
pH	6.88	**7.45**	6.78
COND	0.43	0.46	**1.17**
TOC	35.22	**45.67**	35.18
Na+	7.96	10.44	**90.62**
K+	0.62	6.11	**8.72**
NH4+	0.66	0.88	**4.51**
Ma2+	5.01	4.00	**21.24**
Ca2+	28.35	36.46	**52.62**
F-	0.10	**0.94**	0.34
Cl-.	17.13	22.12	**188.08**
NO2-	0.77	**0.90**	0.27
Br-	0.48	0.24	**1.43**
NO3-	4.21	2.39	**7.86**
PO43-	0.20	0.46	**1.70**
SO42-	27.45	32.81	**116.63**
BLINH	**−14.91**	−28.67	−20.00
MORT	7.32	**14.79**	10.19
GRINH	2.32	**8.68**	−0.05
Cd	0.004	**0.039**	0.004
Cr	**0.074**	0.003	0.058
Co	0.099	0.018	**0.112**
Fe	0.74	0.73	**0.76**
%DNA	11.82	**25.54**	16.84

3.2. Results of PCA

Four latent factors (PC1-PC4) are responsible for explanation of nearly 60% of the total variance. In Fig. 2, the plots for the factor loading for each PC are presented.

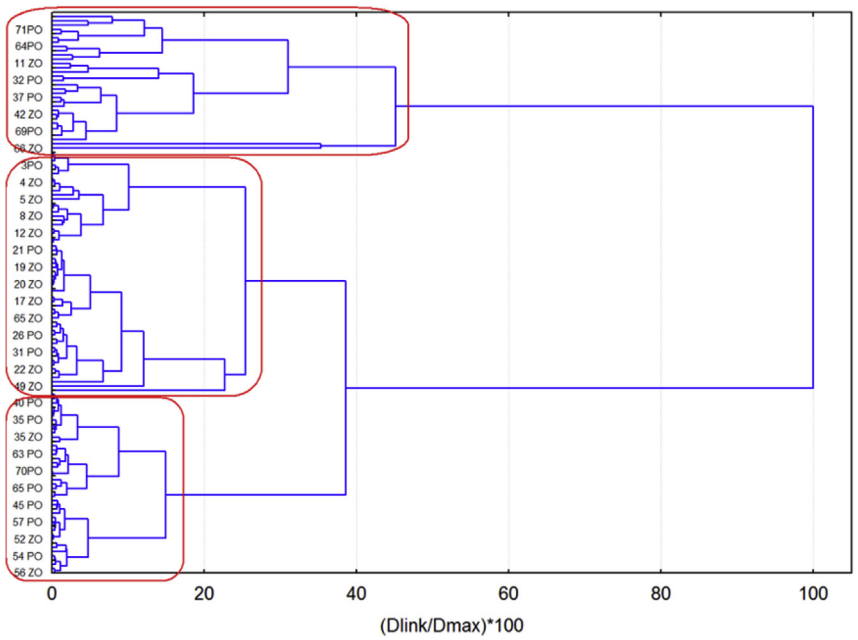

Fig. 1. Hierarchical dendrogram for clustering of WWTPs (all locations – each fourth point is shown in the figure).

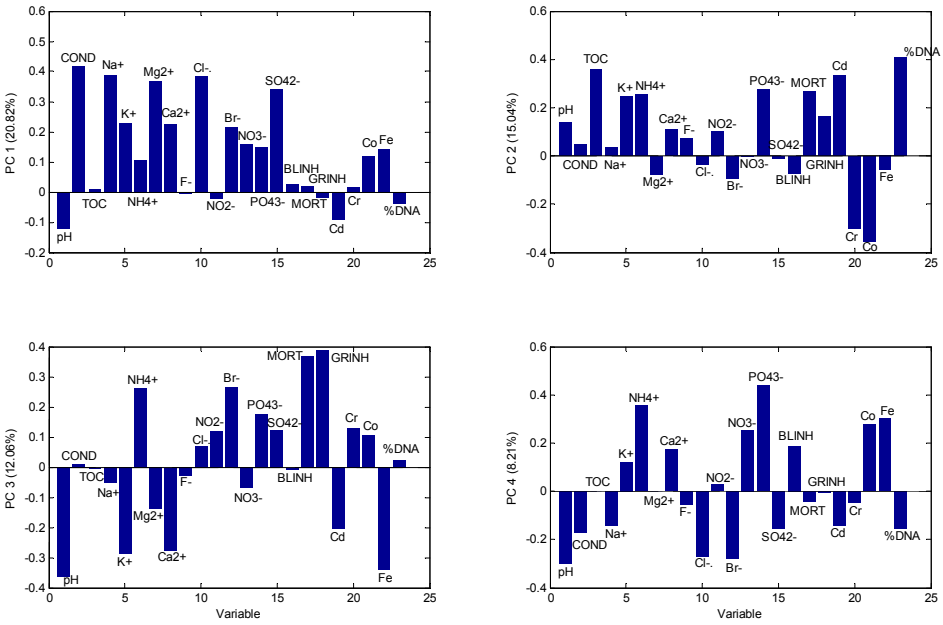

Fig. 2. Factor loadings plot for 4 latent factors.

The four latent factors are responsible for explanation of nearly 60% of the total variance.

The first latent factor (PC1) explains over 20% of the total variance. It could be conditionally named the ***"total inorganic pollution"*** factor because it indicates a strong correlation of conductivity with all other chemical parameters. No correlation is found with ecotoxicity tests, TOC, pH and DNA disruptors.

The second latent factor (PC2) with over 15% of the total variance reveals a strong correlation between TOC, DNA disruptors, pH and Cd. It could be conditionally named the ***"organic toxic effluents"*** factor as it combines the impact of toxic pollutants on DNA of the test organisms. Obviously, organic pollution impact is related to ecotoxicity for tests in which DNA disruptors are responsible.

The third latent factor (PC3) explains over 12% of the total variance of the system and its conditional name could be the ***"ecotoxicity"*** factor due to its significant factor loadings of the mortality (MORT) and growth inhibition (GRINH) of *Heterocypris in*.

The last factor (PC4), with over 8% of the total variance, indicates the role of pollution caused by agricultural activity (strong correlation between typical soil nutrition components such as phosphate, nitrate and ammonia). Its conditional name could be the ***"soil nutrition agricultural"*** factor. It is worthwhile to note that the bioluminescence inhibition test (BLNH) is also significantly correlated to the major group characteristics with high factor loadings that determine the soil nutrition impact on water pollution.

An important conclusion from the PCA is that the different ecotoxicity tests appear to be related to different pollution impacts and it determines their specific applicability to different sources of chemical pollution. It is always a difficult task to select organisms for batteries of biotests/bioassays. Here, an attempt was made to reflect response at both the molecular level (comet assay), and the bacterial and crustacean levels. Although *Heterocypris incongruens* is not organism of "first choice" there are many advantages to this approach: it is less sensitive to highly polluted samples than *Daphnia* sp. or *Thamnocephalus* sp., and for this reason, a more unified response can be obtained for the entire dataset.

Interesting observations were made after ordering all WWTPs results (in modes ZO and PO) according to the factor scores for each one of the identified latent factors (the new coordinates of the objects which correspond to the original variables in the initial data set). In Fig. 3(a–d), factor score plots for each latent factor are presented.

The spatial spread is significant with respect to PC1. This is a logical result because this latent factor represents the general level of pollution being different at different locations. However, few sites are outliers, as the range of possible change along 0 for PC1 is quite large. It is worth mentioning that among 8 strongly inorganic-polluted sites, only two WWTP (32 – Katowice and 64-Kraków Balice) input and output locations are involved.

For PC 2 (organic pollution impact assessed for ecotoxicity by DNA disruptor test) even fewer sites prove to be outliers. Along with the output of Katowice WWTP (32 ZO), outliers are the output sites of WWTPs from the north-eastern part of Poland (Kętrzyn, Nowa Wieś Ełcka, Bielsk Podlaski and Łapy). For PC 3, there is a relatively homogeneous "ecotoxicity" pattern with as the same outlier as in PC1 and PC2: Katowice WWTP (32 ZO).

Finally, PC4 indicates a homogenous "soil nutrition". An interesting issue is that Katowice WWTP shows the only specific behaviour. Input location (Katowice) has the minimum factor score and the output location (Katowice) the maximal ones.

To distinguish in a more detailed way the outliers (representative for the positive or negative impact of the WWTP on the water quality, i.e., for the improvement or deterioration of the water when entering and leaving the plant), the following plots of cumulative site frequency distribution (based on the difference PO – ZO factor scores for each WWTP) for each of the identified latent factors are shown (Fig. 4 a–d). The locations specified on the upper right side of each plot are locations showing improvement after leaving the WWTP (lower level of chemical content or ecotoxicity results) because those indicated on the down left position of the plot indicate deterioration of the quality.

Noticeable change of water body condition could be observed in several cases when assessed with different bioassay as presented in Table 3.

It can also be concluded that some non-determined parameters affecting impact of pollutants on bioassay organisms exist, as in several cases, the same parameters positively impacting them, and in other cases, it is in a negative way (as summarized above).

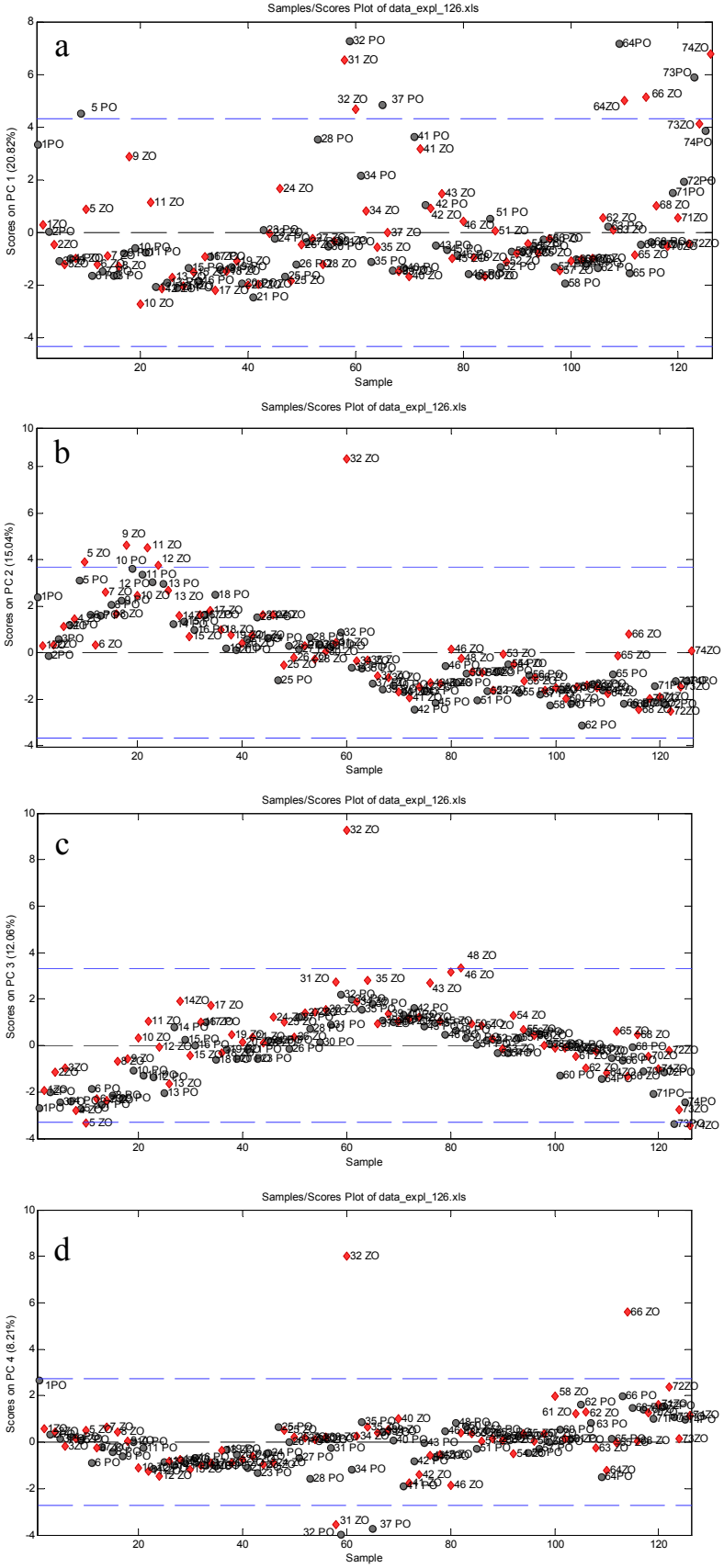

Fig. 3. a. Factor scores plot for PC 1. b. Factor scores plot for PC 2. c. Factor scores plot for PC 3. d. Factor scores plot for PC 4.

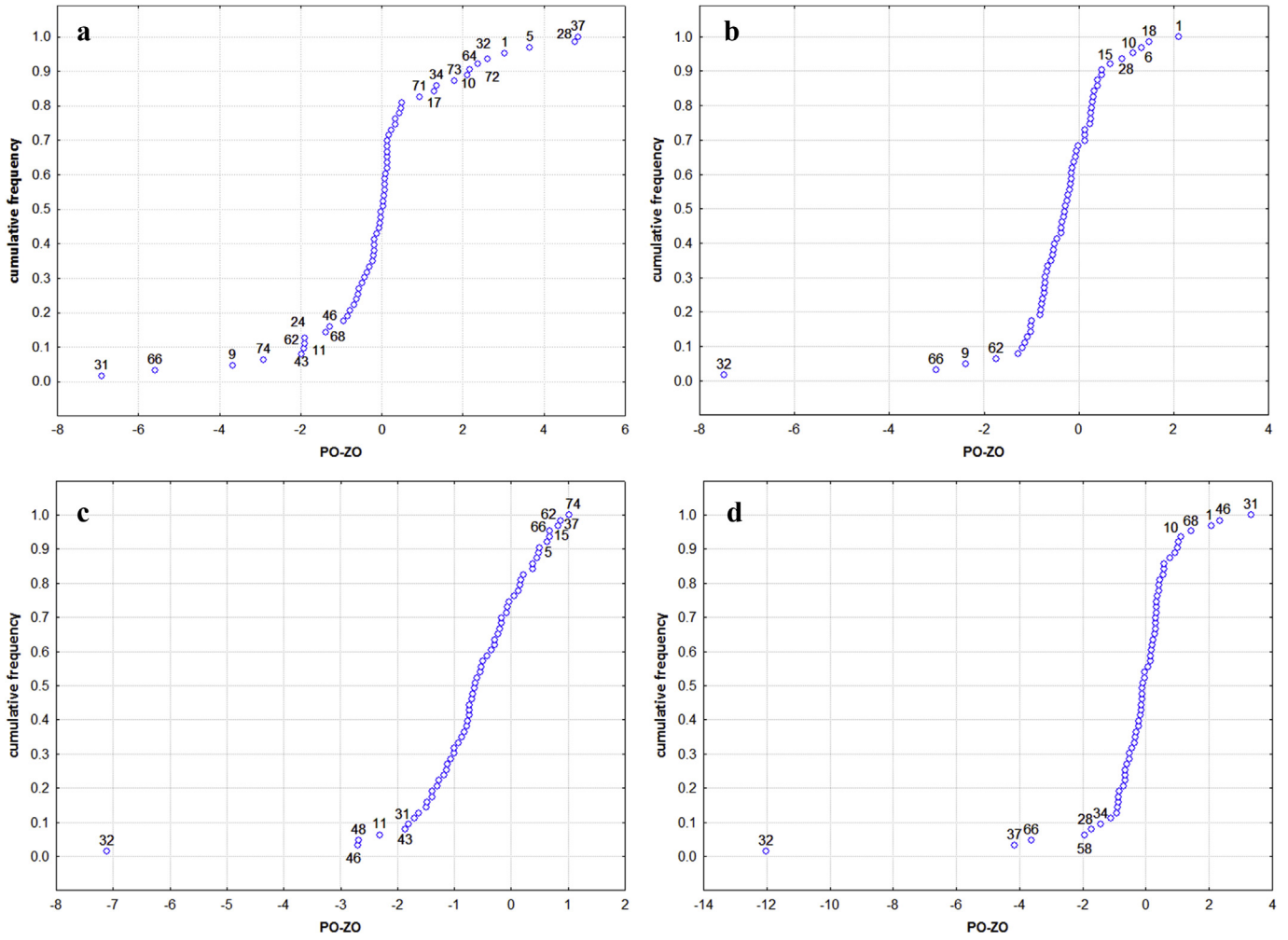

Fig. 4. a. Cumulative frequency for locations with respect to PC1. b. Cumulative frequency for locations with respect to PC2. c.Cumulative frequency for locations with respect to PC3. d. Cumulative frequency for locations with respect to PC4.

Table 3
List of locations negatively and positively impacted by WWTP in regards to PCA.

Water quality deteriorating WWTP	Water quality improving WWTP
PC1: Sosnowiec, Mielec, Grajewo, Żory, Wrocław, Bielsk Podlaski, Oświęcim, Łódź, Staszów Połaniec, Ciechanów	PC1: Zdzieszowice, Radomsko, Kętrzyn, Elbląg, Katowice, Kielce, Kraków, Białystok, Zielona Góra, Chorzów, Police, Ostrowiec Świętokrzyski
PC2: Katowice, Mielec, Grajewo, Oświęcim	PC2: Elbląg, Stargard Szczeciński, Giżycko, Białystok, Radomsko, Kołobrzeg
PC3: Katowice, Ciechanów, Siedlce, Bielsk Podlaski, Wrocław, Sosnowiec	PC3: Żory, Zdzieszowice, Oświęcim, Kołobrzeg, Mielec, Kętrzyn
PC4: Katowice, Zdzieszowice, Mielec, Sanok, Radomsko, Chorzów	PC4: Sosnowiec, Ciechanów, Elbląg, Staszów Połaniec, Białystok

Additionally, in some situations, the same WWTP deteriorates the water quality for a certain PC (type of pollution) but for other PCs improves it (Sosnowiec, Ciechanów, Mielec, Żory, Zdzieszowice, Katowice, Nowy Targ, Radomsko, Chorzów, Oświęcim, and Staszów Połaniec). It might mean that different WWTP are impacted differently by polluting agents and react distinctly to different types of pollution. Studies in this field will be continued.

4. Conclusions

The present study aimed to assess the possibility of utilizing biotests in evaluating the efficiency of pollutant removal in industrial and municipal WWTPs of Poland. Both classical and biological (Microtox®, Ostracodtoxkit F™ and comet assay) methods were used to determine the environmental impacts of certain WWTPs. Interestingly, in some cases, water quality improvement indicated as a toxicity decrement toward one of the bio-indication organisms makes the water quality worse for others. This fact is particularly noticeable in the case of Polish Silesian cities and those in the vicinity of large chemical plants (e.g., Police) where heavy industry and high population density is present. It proves that each WWTP should undergo an individual evaluation of pollutant removal efficiency and be tuned to selectively remove pollutants of highest risk to surrounding regional ecosystems. Biotests again proved to be an extremely important tool to fully assess the impact of environmental stressors on water bodies receiving effluents from WWTPs, and the biotest battery selection is of significant importance.

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Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.06.086.

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