Development of a Bioelectronic Tongue -Applications for Wastewater Analysis

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Chemometric exploration of an amperometric biosensor array for fast determination of wastewater quality

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Abstract

Four wastewater samples of different treatment qualities; untreated, alarm, alert and normal, from a Swedish chemi-thermo-mechanical pulp mill and pure water were investigated using an amperometric bioelectronic tongue in a batch cell. The aim was to explore enzymatically modified screen-printed amperometric sensors for the discrimination of wastewater quality and to counteract the inherent drift. Seven out of eight platinum electrodes on the array were modified with four different enzymes; tyrosinase, horseradish peroxidase, acetyl cholinesterase and butyryl cholinesterase. At a constant potential the current intensity on each sensor was measured for 200 s, 100 s before injection and 100 s after injection of the sample. The dynamic biosensor response curves from the eight sensors were used for principal component analysis (PCA). A simple baseline and sensitivity correction equivalent to multiplicative drift correction (MDC), using steady state intensities of reference sample (catechol) recordings, was employed. A clear pattern emerged in perfect agreement with prior knowledge of the samples explaining 97% of the variation in the data by two principal components (PCs). The first PC described the treatment quality of the samples and the second PC described the difference between treated and untreated samples. Horseradish peroxidase and pure platinum sensors were found to be the determinant sensors, while the rest did not contribute much to the discrimination. The wastewater samples were characterized by the chemical oxygen demand (COD), biological oxygen demand (BOD), total organic carbon (TOC), inhibition of nitrification, inhibition of respiration and toxicity towards *Vibrio fischeri* using Microtox®, the freshwater alga *Pseudokirchneriella subcapita* and the freshwater crustacean *Daphnia magna*.

Keywords: Wastewater; Pattern recognition; Principal component analysis; Multiplicative drift correction; Bioelectronic tongue; Amperometric sensor array

1. Introduction

Fast, reliable and continuous monitoring of wastewater has to be established in the coming years in order to manage discharges to the environment of potentially harmful wastes. It is necessary to develop discharge-monitoring systems that at any given moment, i.e. in seconds or minutes, can indicate whether discharges are of an appropriate quality or whether an incident of treatment plant failure is underway. Today wastewater monitoring is limited to chemical and biological testing, which takes hours or even days to perform. Especially biological testing is very time-consuming, expensive and indeed limited by the test itself and thus not applicable

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in alarm system. An overview of different types of on-line chemical sensors and principals used in wastewater monitoring and control are given in (Lynggaard-Jensen, 1999). Most sensors are based on univariate recording of data however more and more attention has recently been directed to the use of multivariate sensors. This can be realized by using instruments measuring in more than one channel, e.g. spectroscopic instruments or by measuring different or similar properties simultaneously with more than one sensor, i.e. sensor arrays. An intelligent sensor system, delivering a continuous fingerprint that is processed in a computer with a chemometric method such as principal component analysis (PCA) or partial least squares regression (PLS-R), could thus be trained to respond to pattern deviations from the normal situation.

Electrochemical sensor arrays for measuring physical, chemical or biological properties in gas phase or in liquid phase are often referred to as electronic noses (Strike et al., 1999) and electronic tongues (Krantz-Rulcker et al., 2001), respectively. The great advantage of electrochemical sensors is the high sensitivity and for some also high specificity (Stefan et al., 1999). Biologically modified sensors mimic biological processes on the surface and may thus in the future become widespread and specifically identify certain compounds or groups of compounds known to be harmful. Depending on the physical sensor setup, i.e. sampling device, flow injection–contra steady state measurements, results in the form of an alarm indication would appear in no more than a few minutes or even in seconds. The relatively low costs of equipment (€∼1500), may also prove advantageous when implementing these techniques for real-time monitoring in various environments. The sensors and the combination of sensors used in the arrays are of great variety both in properties measured, in stability, in the data recording and in the subsequent data analysis. Recording multivariate dynamic responses over time with many sensors simultaneously is, however, only now being explored. Common for the data analysis is the multivariate pattern recognition approach using various linear or nonlinear modelling like principal component analysis (PCA) (Artursson et al., 2000; Delpha et al., 2001), discriminant factor analysis (DFA) (Delpha et al., 2001), partial least squares regression (PLS-R) (Artursson et al., 2000; Plegge et al., 2000; Delpha et al., 2001), artificial neural networks (ANN) (Delpha et al., 2001), nonlinear multivariate calibration (Plegge et al., 2000), etc. The data structure of nose and tongue measurements have similar properties, as a result, it is often possible to apply ideas developed in one area in the other.

In this study, the use of a sensor array with eight amperometric sensors modified with four different enzymes was explored for wastewater quality determination (Fig. 1). Each sensor returns a dynamic time response curve at a fixed potential upon injection of sample. Only few studies of dynamic sensor response curves employing multivariate data analysis can be found in the literature. Three papers are concerned with simultaneous analysis of binary and ternary mixtures using amperometric microbial (Slama et al., 1996; Plegge et al., 2000) or enzyme (Dock et al., 2004a,b) based sensors, while a fourth is concerned with quantitative gas detection under varying environmental conditions (Delpha et al., 2001). The latter uses feature extraction like slope and steady state intensity from conductivity curves.

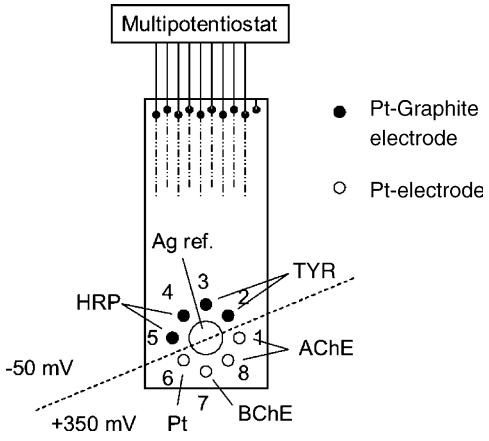

Fig. 1. Biosensor array with immobilized enzymes connected to a multipotentiostat; Pt–graphite sensors 2 and 3 with tyrosinase (TYR); Pt–graphite sensors 4 and 5 with horseradish peroxidase (HRP); Pt sensor 6 was left unmodified (Pt); Pt sensor 7 with butyryl cholinesterase (BChE); Pt sensor 8 and 1 with acetyl cholinesterase (AChE). A potential of −50 mV and 350 mV relative to the Ag/AgCl reference electrode was applied to Pt–graphite and Pt electrodes, respectively.

Sensors based on biological responses from immobilized microorganisms and enzymes are inherently plagued by temporal drift. The drift is caused by a variety of factors determined by the chemical and physical environment, i.e. temperature, pH and other constituents, which may influence the signal both in the short and in the long term (aging). Mathematical methods to counteract the drift includes additive correction (Holmin et al., 2001), multiplicative drift correction (MDC) (Artursson et al., 2000; Haugen et al., 2000) and component correction (CC) (Artursson et al., 2000; Holmin et al., 2001) and involves measuring known substances or mixtures of substances as reference. The additive correction (Holmin et al., 2001) (i.e. baseline correction) works if there is a drift in all sensor/channel responses towards a constant matrix present in the same amount in all samples or in cases where the instrument drift is only concerned with the baseline and not the sensitivity. If the sensitivity is influenced by drift, MDC or similar methods should be applied. In MDC it is assumed that the drift is multiplicative, which means that the perturbation from poisoning, environmental changes or aging is proportional to the signal level. Also the relationship between the response to the reference sample and the response to the real sample must be linear, and a high signal to noise ratio in the reference measurements is important for stable corrections. CC is highly inspired by orthogonal signal correction (OSC) (Wold et al., 1998; Eriksson et al., 2000) and is aimed at removing the irrelevant information in data by defining the direction of the drift by PCA of the stan-

dard measurements and then removing this direction from the responses of the samples. The advantages are that the drift does not have to be linear as long as it is in the same direction both for samples and for reference measurements and indeed sensors with low signal to noise ratio do not influence the direction much as their importance is low in the PCA. This can work well as long as the direction of the drift described by the drift in the reference sample is different from direction(s) of relevant information. This, thus, presumes that the relationship between similar sensors and/or channels is linear and that this relationship is the same for reference samples and for the real samples. CC have in some cases proved to be better than MDC (Artursson et al., 2000), but will not be presented in this paper, as the information in the present data at least for some of the sensors lies in the same direction as the drift, i.e. has similar loadings. This may often be the case in real life applications. Removing this component from the sample measurements would thus impair the information in data. For a thorough discussion of these two correction methods, please refer to Artursson et al. (2000). A reference sample is indeed necessary for these correction methods, but should be chosen with some care. The assumptions made for calibration demands that the reference signal is highly correlated with the real sample signals to be measured, i.e. the sensors should respond to the reference and give signals in the same order of magnitude as the real samples. Other methods that do not require reference samples can be found (Holmberg et al., 1996, 1997) and further development of the analytical system itself and the use of flow injection systems with different drift compensating cleaning steps may also be feasible (Winquist et al., 2002). However, drift seems to be an unavoidable challenge that requires thorough attention and validation in each case. In this paper, the simple baseline and sensitivity correction is used on a sensor-to-sensor base as the different sensors drift individually.

2. Materials and methods

2.1. Wastewater samples

Untreated wastewater from a Swedish CTMP mill was aerobically treated in a laboratory scale wastewater treatment plant (WWTP) situated at the Swedish company, ANOX in Lund. The WWTP had a treatment capacity of 2–3 L of wastewater per day. One sample of untreated wastewater (UN) was taken directly from the bulk before initiation of treatment. Three composite samples were collected over three periods of approximately 1 month each with stable conditions producing 20 L of wastewater with specific assigned quality; alarm (AL), alert (AT) and normal (NO), respectively, according to the level of chemical oxygen demand (COD). The treatment quality was controlled by addition of nutrients N and P to the wastewater prior to the treatment and monitored by measuring the COD every 2–4 days. For blank sample pure tap water (WAT) was used. In all five different samples of different treatment quality was employed in the sensor array investigation.

2.2. Chemical and biological tests

The following 10 biological and chemical tests were made to characterize the wastewater samples: (1) Microtox® luminescence inhibition test of the freeze dried marine bacteria *Vibrio fischeri* strain NRRL B-11177 according to the ISO 11348-3 (1998) and the Microtox model 500; Microbics Corp. with an automatic registration of the luminescence. The samples were adjusted to pH 7.0 ± 0.2, if necessary. The 50% inhibition of the luminescence (EC50) after 15 min of incubation at $15 \pm 1\,^{\circ}C$ was determined by using the computer program of Microtox Acute Toxicity Test, Azur Environmental Ltd., UK; (2) growth inhibition test with the freshwater alga *Pseudokirchneriella subcapita* according to ISO 8692 (1989). Fifty percent growth inhibition (EC50) of the algae population was determined after 72 h of exposure at a temperature of $23 \pm 2\,^{\circ}C$ and pH 7.3–9.1. EC50 was determined by use of the computer program TOXEDO (VKI 1999); (3) acute static toxicity test with the freshwater crustacean *Daphnia magna* according to ISO 6341 (1996) was used for determination of the IC50 for immobilisation of daphnia after 48 h of exposure at a temperature of $20 \pm 2\,^{\circ}C$ and pH 6.9–8.8. The IC50 was calculated by using a Probit program (SNV 1992); (4) inhibition of nitrification according to SS-EN ISO 9509 (1989); (5) inhibition of respiration according to SS-EN ISO 8192 (1986); (6) COD according to Dr. Lange LCK 114; (7) BOD according to SS-EN ISO 1899-1; (8) TOC according to SS-EN 1484; (9) pH; (10) conductivity at $25\,^{\circ}C$.

2.3. Chemicals for sensor array

The enzymes tyrosinase (TYR, EC 1.14.18.1, Sigma cat. No T-7755, $6050\,U\,mg^{-1}$), acetyl cholinesterase (AChE) from electric eel (EC 3.1.1.7, Sigma cat. No C-3389, $292\,IU\,mg^{-1}$), butyryl cholinesterase (BchE) from equine serum (EC 3.1.1.8, Sigma cat. No C-1057, $345\,IU\,mg^{-1}$) and horseradish peroxidase (HRP, EC 1.11.1.7, Boehringer Mannheim GmbH cat. No 814407) were used without further purification. Acetylthiocholine chloride (ATCh-Cl), bovine serum albumin (BSA), glutaraldehyde (GA) and catechol were purchased from Sigma–Aldrich, Steinheim, Germany. Hydrogen peroxide (30%) was purchased from Merck, Darmstadt, Germany. A 0.05 M, pH 7 phosphate buffer (PB) with 0.1 M potassium chloride was prepared from analytical-reagent grade reagents from Merck in water obtained from Millipore Milli-Q purification system (Bedford, MA, USA). A 1% glutaraldehyde solution was prepared with PB as solvent. Likewise solutions of enzymes for sensor array preparations (below) were prepared using PB as solvent.

2.4. Sensor array preparation

A sensor array from BVT Technologies a.s., Tisnov, Czech Republic consisting of eight screen printed platinum working electrodes, 1 mm in diameter and arranged radially around a common silver reference electrode was modified as follows (Fig. 1). The working electrodes were printed with carbon paste DP7101 (Dupont, USA), however, on four of the screen printed electrodes the graphite layer was removed using an acetone:cyclohexanone mixture (1:1). The enzyme immobilisation procedures were executed according to the following (Solná et al., 2005): TYR and HRP were immobilized by cross-linking with glutaraldehyde. The immobilization mixture was prepared from 33 μL of the enzyme solution (10 mg mL^{-1}), 10 μL of phosphate buffer and finally 13 μL 1% glutaraldehyde was added as the last component. For the immobilisation of the cholinesterases the following procedure was used: mixtures were prepared from 20 μL of AChE or BChE solution (20 nkat μL^{-1}, i.e. 18.5 mg μL^{-1} and 4.1 mg μL^{-1}, respectively), 24 μL of bovine serum albumin (50 mg mL^{-1}), 220 μL of water, 44 μL of phosphate buffer, and finally 26 μL 1% glutaraldehyde, added as the last component. All the above enzyme mixtures were freshly prepared and used immediately. Less than 1 μL of the enzyme mixtures were added to the surfaces of the electrodes so that the electrode was completely covered by the enzyme solution and at the same time did not mix with neighbouring electrode solutions. TYR was applied to the surface of Pt–graphite electrodes 2 and 3, HRP was applied to Pt–graphite electrodes 4 and 5, AChE was applied to Pt electrodes 1 and 8, BChE was applied to Pt electrodes 7, while Pt electrode 6 was left unaltered (Fig. 1). The resulting biosensor array was stored over night at 5 °C in a Petri dish sealed with para film. The modified array was rinsed with Milli-Q water and dried in air. The graphite electrodes were then covered with drops of PB solution and the array was stored at 5 °C along with a PB moistened tissue in the sealed Petri dish until usage.

2.5. Recording dynamic response curves

The sensor array was placed in an electrochemical batch cell with a rotating rod at a speed of 15 Hz (Dock et al., 2004a,b) and connected to an eight channel multi-potentiostat from Prof. J. Kulys, Laboratory of Enzyme Chemistry, Institute of Biochemistry, Vilnius, Lithuania, which was connected to a PC. The data were collected using the software program Intels 1.5, specially created for the potentiostat. A potential of −50 mV and +350 mV relative to the Ag/AgCl reference electrode was applied to the graphite and Pt electrodes, respectively (Fig. 1). The 4.50 mL PB spiked with 0.553 mM acetylcholine chloride, 16.6 μM catechol and 55.5 μM H_2O_2 was placed in the cell and measurements were initiated. After 100 s lag-time 0.5 mL sample was manually injected and recording was continued for 100 s. In all 820 time channels were recorded for each sample. The cell was then emptied and rinsed with water before repeating the procedure for a new sample. In all, 20 measurements were carried out. The five samples (UN, AL, AT, NO, WAT) were measured four times each in randomized order and after each two samples, instead of the sample 0.5 mL, 0.15 mM catechol was injected after the initial lag-time to realize the reference measurement. In all, 12 reference–response curves were recorded.

2.6. Drift correction and chemometric analysis

Of the original three-way $32 \times 8 \times 820$ matrix (32 samples/references, 8 sensors, 820 time channels), 400 time channels were selected from each sample recording starting at 50 channels before injection of sample, thus reducing the matrix to $32 \times 8 \times 400$. All samples were thus aligned with respect to the injection point (Fig. 2a) by manually defining the time of injection in each individual recording based on the first systematic change in sensor response. The average signals of channels 10–40 for each sensor was defined as baseline for each individual measurement and subtracted from the entire response curve of each particular sensor, thus removing the offsets (Fig. 2b). In order to correct for sensitivity drift, all response curves were divided with the mean of the steady state signals in time channels 100–400 in the response curve of the catechol measurement recorded immediately before or after the sample recording (Fig. 2c). In MDC, the curves would again be multiplied with the steady state signal of the first catechol measurement made to convert the units back to current intensity, but in this case the change of unit has no practical implications.

By removing the catechol measurements and unfolding the eight simultaneously measured and normalized response curves an X matrix of dimension 20×4000 were made for the chemometric analysis. Thus starting from column one, for every 500 channels a new sensor response of 400 channels was represented followed by 100 missing values. The missing values were solely for better visual separation of the sensors, when plotting the responses (Fig. 2a–c). For each sensor the time channels from the point of injections was used in principal component analysis in The Unscrambler® software Version 7.6 SR-1, CAMO A/S, Trondheim, Norway. Two measurements of the untreated sample were detected as outliers and removed during the analysis. This was probably due to the combination of the extreme nature of the untreated sample itself and that the measurement were made early in the series, where the drift in sensor array sensitivity was too rapid to be accounted for by reference measurements.

3. Results and discussion

3.1. Sample characteristics

An overview of the sample characterization is shown in Table 1. The COD, BOD and TOC content of the wastewater samples clearly demonstrate the degradation of organic mate-

rial obtained at the different levels of treatment as assigned by the quality terms: untreated, alarm, alert and normal. The levels of treatment did also affect the toxicity of the wastewater. Thus, the results of the ecotoxicity tests show a decreasing

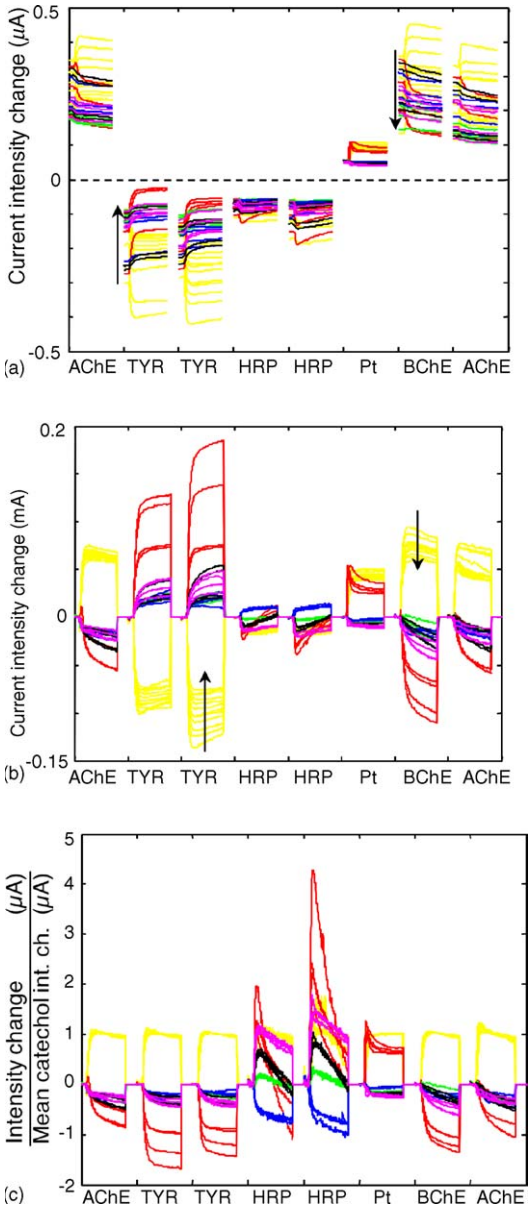

Fig. 2. The eight sensor response curves arranged consecutively of 400 time channels separated with 100 missing values. In all 32 wastewater and catechol recordings were made with replicate colour scheme; untreated (red), alarm (magenta), alert (black), normal (green), water (blue) and catechol (yellow). (a) Raw response curves of current intensity aligned to point of injection after 50 time channels. The arrows indicate the drift in offset with time seen in all sensors except the unmodified Pt electrode. (b) Baseline corrected response curves, thus representing the current intensity change induced by injection of sample or catechol reference. The arrows indicate the drift in sensitivity with time towards catechol. (c) Baseline and sensitivity corrected response curves represented by the relative current intensity change to steady state catechol intensity at the time of measurement, thus catechol curves are all forced to be one. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 1
Wastewater samples characterized by various chemical and biological tests

Sample	pH	Conductivity (mS/cm)	COD (mg/L)	BOD (mg/L)	TOC (mg/L)	Inhibition of nitrification effect at 400 mL/L (%)	Inhibition of respiration EC20	Microtox EC50 (mL/L)	Algae EC50 (mL/L)	Daphnia IC50 (mL/L)
UN	6.6	5.47	7600	3500	3100	38	>400	3.0	43	29
AL	8.39	5.96	4100	600	1600	24	>400	17.6	84	276
AT	8.48	5.74	2400	250	1100	13	>400	174	30	>500
NO	8.57	6.39	2200	160	850	16	>400	>500	88	>500
WAT	Not measured	Not measured	Not measured	Not measured	Not measured	0	>400	>500	>500	>500

toxicity towards *Vibrio fischeri* and nitrifying microorganisms by increased treatment efficiency. However, the toxicity towards algae was still quite high for the 'alert' and 'normal' sample and not coherent with the assigned quality terms. This indicates that algae toxicants were present at a high level in the 'normal' wastewater sample and perhaps even produced during the degradation in the WWTP resulting in an EC50 at 30 in the 'alert' sample. It is noteworthy, that inhibition of respiration in activated sludge could not be detected in either of the samples at concentrations above 400 mL/L and that the effect towards *Daphnia magna* was very significant in the untreated sample but vanished in the 'alert' and 'normal' samples. The results of the sample characterization clearly show that measuring organic material only by COD, BOD or TOC does not give satisfactory information about the possible effects of the wastewater in the aquatic recipient. An assessment of potentially hazardous wastewater should, therefore, include toxicity-based measurements. Several different biological tests are often required due to the complexity of the ecosystem and the possible different modes of toxic activity by the constituents in a complex mixture such as wastewater. Biological tests are often time-consuming and it is not realistic to use a set of biological test batteries as part of an alarm system for a continuous wastewater outlet. It certainly calls for more rapid and inexpensive methods to be developed, that can serve as alarm indicator for wastewater, which is harmful to the recipient, e.g. indicating changes in COD, TOC, BOD, N or P levels as well as toxicity changes towards bacteria, algae, crustacean or fish. These methods would instantly indicate possible changes in the treatment quality or incidents of treatment plant failure for a timely preventive action to be taken.

3.2. Raw data

Fig. 2a presents the raw data aligned according to point of injection. The first 50 time channels on each sensor are the last 12 s of the initial 100 s of recording on the catechol spiked buffer solution and may be assumed to be the steady state signal before the injection of sample or reference solution. These signals are positive (i.e. oxidation currents) for the sensors with positive potential, i.e. sensor 1, 6, 7 and 8 with AChE, BChE and pure Pt (Skladal et al., 1997; Sapelnikova et al., 2003), while they are negative (i.e. reduction currents) for the sensors with negative potential, i.e. sensors 2–5 with TYR and HRP (Marko-Varga et al., 1995; Sapelnikova et al., 2003). Detailed information about sensor array functionalization as well as sensor array reproducibility and performance was recently reported in (Solná et al., 2005). When the standard solution (catechol) is injected the signals increase rapidly in either positive or negative direction (yellow lines in Fig. 2a), depending on the type of sensor studied. For TYR and HRP based sensors catechol functions as a mediator that shuttles electrons between the enzyme product and the electrode surface (Marko-Varga et al., 1995; Sapelnikova et al., 2003). The catechol currents obtained at the other sensors are due to direct oxidation at the electrode surface. The catechol concentration was chosen so that the current signal showed a linear dependence for all sensors in the experiments. When wastewater samples are injected the current intensities decrease, indicating an inhibition of the sensors towards the catechol already present in the buffer. This shows that even though the wastewater may contain compounds that presumably could increase the current intensity of the sensors, e.g. phenols such as tannins and various degradation residues, the inhibiting effect is much stronger. This effect of the wastewater constituents on the sensor performance is not easily characterised, but may be due to the harmful hydrophobic resin and fatty acids known to be present in CTMP wastewater and also known to be responsible for the major toxicity (Ali and Sreekrishnan, 2001). The presence of catechol in the buffer solution was indeed motivated by the fact that a complex matrix such as wastewater might increase as well as inhibit the sensor signal. Without catechol in the buffer the effect of the wastewater injection would probably not be as dramatic as is seen here.

3.3. Drift correction

The primary goal of this investigation was to determine whether a set of biosensors would be able to give meaningful responses corresponding to the quality of the wastewater treatment, i.e. qualitative information. From the raw data in Fig. 2a, it is indeed quite difficult to discriminate different samples due to the decreasing sensitivity with time. After one full day of 32 recordings all sensors were still functional, however, with a significant decrease in sensitivities. This instability is due to that the sensors are influenced both by mechanical and chemical factors of the setup, in particular the sample composition, which in this case is very complex. A drift in offset and sensitivity and probably also in response pattern is observed and must be addressed mathematically in order to use the data. In Fig. 2a–c, this conversion of data is shown. In Fig. 2a different offsets are clearly observed represented by the different steady state signals before injection of the samples or reference solution. This is true both between individual sensors and within each sensor. By removing the offset (Fig. 2b), i.e. subtracting the steady state signal from each individual sensor response curve for all the samples, the additive drift is eliminated. Still a drift in sensitivity of some sensors, especially in TYR, AChE and BChE, are quite significantly observed in Fig. 2b by the fact that measurements of identical samples or reference solution give decreasing responses with time. Normalizing to the responses of the nearest catechol measurement clearly increase the value of information in the dynamic response curves (Fig. 2c). Each replicate measurement for the individual samples is now arranged on top of each other and thus looks as if discrimination between the samples, i.e. grouping of replicate measurements, is possible just by inspecting the drift corrected biosensor measurements in Fig. 2c. The TYR, HRP and AChE sensors, which are all present in duplicates, show comparable signals

after correction. This is encouraging when considering manual preparation of the individual sensors, i.e. adding a drop of enzyme mixture on top of the individual electrodes. This confirms that the redundant information found in each time channel in every individual sensor response curve is highly correlated, enabling this very simple correction to work quite well. However, this correction does not counteract the drift in curve shape, which can be very significant with these types of sensors. For such a correction every time channel or the average of several time channels should undergo MDC as done by Dock et al. (2004a,b). This method was not applied here due to fact that the present data were recorded using manual injection and a manual aligning procedure, which all together produces artefacts and low signal to noise ratios in some channels that would jeopardize the possibility to evaluate and validate the method. The drift in catechol signal is highly correlated to the drift in sample signal. This somewhat contradicts the assumption that the standard solution has to be both chemically similar to the samples and in the same concentration range (Artursson et al., 2000; Haugen et al., 2000), which is probably not the case in this study. But it may be due to the inhibiting effect of the samples on the signal given by the catechol present at all times in the buffer solution. Measurements reflect the samples influence on the catechol signal and thus the above criterion is actually met.

3.4. Principal component analysis

In order to picture the effect of the correction, PCAs of both raw aligned (Fig. 3), baseline corrected (Fig. 4) and sensitivity corrected (Fig. 5) data are presented. Clearly, the score plot of raw data does show some systematic variance according to treatment quality and some grouping. However, the groups of replicate measurements are stretched according to the aging of the sensor array (Arrows in Fig. 3) and discrimination between individual samples become increasingly unclear with the aging of the array. This concludes that

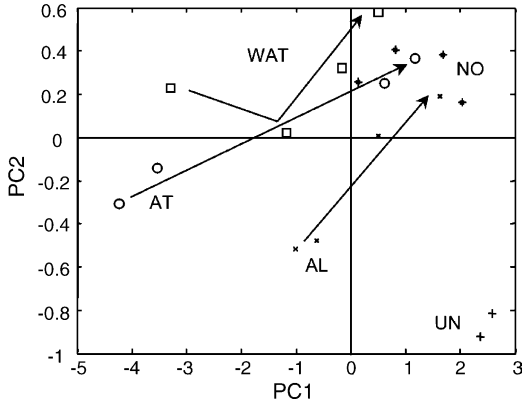

Fig. 3. Scoreplot of PC1 and PC2 of a PCA of 18 raw aligned wastewater response curves (Fig. 2a). (+) Untreated; (×) alarm; (◯) alert; (●) normal; (□) water. The arrows indicate the drift direction with time, thus the first two PCs virtually only captures this drift.

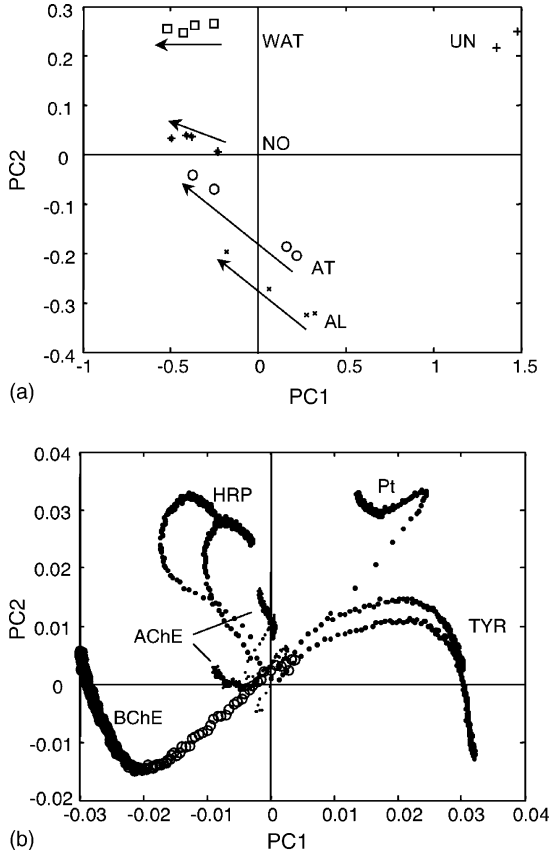

Fig. 4. (a) Scores plot from PCA of 18 baseline corrected wastewater response curves (Fig. 2b). (+) Untreated; (×) alarm; (◯) alert; (●) normal; (□) water. Arrows indicate the directions of the drift. (b) Loading plot indicates that TYR and BChE are negatively correlated, at that they are mainly explaining the drift along the first PC. Pt and BChE explain the difference between untreated and the rest of the samples, while HRP have high influence on the discrimination of AL, AT, NO and WAT.

sensor drift is definitely an issue in the present system. The score plot in Fig. 4a of the baseline corrected data does indeed reveal the information of interest, i.e. a consistent grouping of replicate measurements and clear separation of samples. Still a drift is observed marked by arrows in the plot. Referring to its corresponding loading plot (Fig. 4b) it is seen that TYR and BChE are especially important on the first principle component (PC), which both is the predominant drift direction and the direction separating untreated from the rest, while the HRP is responsible for the variation seen on the second PC, separating the treated samples according to quality. After sensitivity correction the score plot (Fig. 5a) is even clearer in separating the samples. Now the first PC explains the treatment quality of the treated samples with the clean samples to the left and the dirty samples to the right. The second PC reflects that the untreated sample is completely different from the rest. It is important to notice that pattern of clusters in the score plots in Fig. 4a and Fig. 5a is in qualitative agreement with the chemical and biological characterization of the wastewater found in Table 1 and thus validates the correction approach as well as encouraging further studies for fast qual-

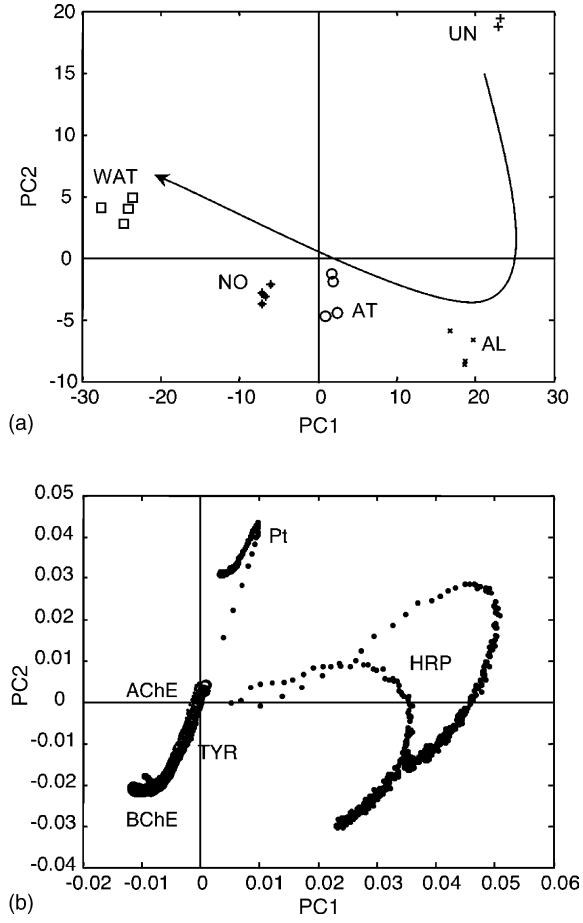

Fig. 5. (a) Score plot from a PCA of baseline and sensitivity corrected wastewater response curves (Fig. 2b). (+) Untreated; (×) alarm; (◯) alert; (●) normal; (□) water. The arrow indicates increasing treatment quality. (b) Loading plot showing that the HRP and Pt sensor have the highest influence on discrimination wastewater quality with the first two PCs explaining 97% of the variance in the data. AChE, BChE and TYR are completely overlapping and do not contribute with any new information in relation to the wastewater quality.

ity determination using enzymatic biosensors. The three consecutive score plots (Figs. 3–5) thus show how appropriate sensitivity correction removes the irrelevant information in the data and reveals the hidden pattern related to the quality of the samples measured. The arrow in Fig. 5 indicates increasing treatment quality. Two principal components (PCs) in Fig. 5 explain 97% of the variance in data validated by full cross-validation. The first PC explains 76% of the data and is the direction related to the treatment quality and the second PC explains 21% of the data and is related to the difference between the treated and the untreated samples. The fact that the major variance in data are related to the knowledge already gained by other means and readily visible by just inspecting the score plot is the strongest validation available, recently referred to as *conceptual validation* (Møller, 2004), which applies when proper chemometric analysis is performed without a priori assumptions about the outcome of the investigation. By examining the loading plot in Fig. 5b it should be noted that the HRP sensor is superior to the other sensors, when it comes to discriminating the samples on the first PC. The pure platinum sensor discriminates well between untreated and the rest, which is probably due to the fact that the positive potential is high enough to oxidize wastewater compounds by direct oxidation. These compounds are presumable not present in any of the wastewater samples, which has been through the aerobic treatment process in the WWTP, as they should already be oxidized. The other sensors do contribute to the second PC also and may actually be relevant in real life in the rare cases where wastewater streams pass untreated through WWTPs.

The AChE (small dots near origo in Fig. 5b) carries virtually no information about the samples while both TYR and BChE do give information about whether a sample is untreated or not on the second PC and thorough study of these sensors individually gives indications of vague clustering in their respective score plots (not shown). To explore the influences of the sensors even further, a PCA with only HRP and Pt sensor data were carried out (Fig. 6a) and compared with a PCA on data from the rest of the sensors (Fig. 6b). The score plot in Fig. 6a is virtually identical to the one in Fig. 5a, where all sensors were used in the calculations. Now the first two PCs represent 85% and 13% of the entire variance. The groups of replicate measurements are even clearer defined. The score plot in Fig. 6b on the other hand does not provide the information of interest. As expected, the untreated versus treated samples are explained by the first PC, however, clear patterns of the other samples do not appear. Continuing working with the HRP sensor with this particular wastewater would be sensible, while the rest may work better in another wastewater. This study thus also shows the possibility to use an array to screen several sensors at the same time for a specific application and help the analyst choose the right sensors.

More work is needed to explore different biological sensors under real conditions, i.e. screening prototype sensors in real wastewater of varying quality. Also, exploration of the predictive capabilities of the sensor for determination of toxicity, BOD, COD and TOC is important for the development of chemometrically stabilized commercial wastewater sensors. Further experiments to evaluate reproducibility between arrays prepared with the same procedure are under processing, using either steady state or flow injection measurements. This work is focused towards finding array compositions that give signals correlating to as many possible toxicity or chemical variables as possible. More investigations must be directed towards the correction of temporal drift under real conditions. Promising results are emerging on using the MDC approach (Haugen et al., 2000) in each time-channel having an appropriate reference with every measurement (Dock et al., 2004a,b). Also, component correction should be tested on larger datasets, but care must be taken as this correction method may destroy information as this may very well lie in the direction of the drift and will thus be erased by this filter.

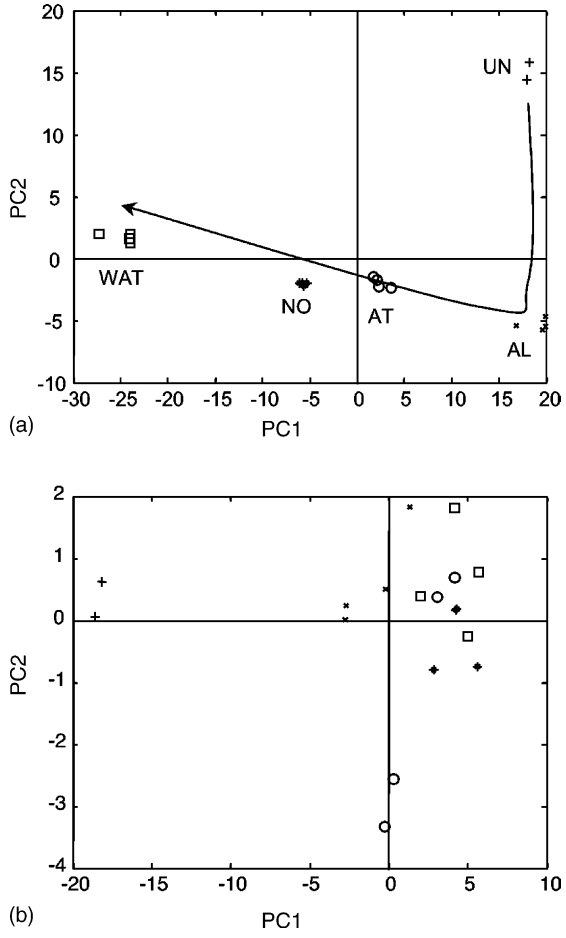

Fig. 6. (a) Score plot from a PCA of wastewater response curves from HRP and Pt sensors alone. This plot is equivalent to the score plot in Fig. 5a with an even clearer separation of the replicate measurements. (b) Score plot from a PCA of data from TYR, AChE and BChE showing that these sensors do not contribute to the discrimination of wastewater samples in the same degree as HRP and Pt. (+) Untreated; (×) alarm; (◯) alert; (●) normal; (□) water.

3.5. Note about aligning

One practical challenge was to define the point of injection as it was based on the first systematic change in response curves due to the actual injection of the sample. The major drawback of this method is the possibility to over interpret and make false assumptions on how and how fast the sensors react to different concentrations of substrate and different samples and thus throw away valuable information. The simple and straightforward way to handle this problem is to include a variable in the data recording controlled by an auto sampler connected to the PC. This devise was not at hand at the time of investigation, but should certainly be used if the aim of fast and reliable detection is the goal. If a reliable injection time cannot be determined, the emerging and encouraging time-channel-wise MDC correction (Dock et al., 2004a,b) is not advisable as it may lead to erroneous correction especially in the first part of the diffusion and kinetic controlled part of the dynamic response curve.

4. Conclusions

In this study, it was demonstrated that four different chemi-thermo-mechanical pulp wastewater samples could be clearly discriminated according to treatment quality using an array of eight amperometric biosensors in combination with principal component analysis. An additive and multiplicative sensitivity correction method for the biosensor data was applied in order to obtain a proper discrimination and thus compensate for the temporal drift in the individual sensors. The pattern obtained by PCA of the sensor response curves was in qualitative agreement with prior knowledge of the samples and chemical as well as biological data obtained, thus validating the overall approach. In addition, it has been shown that the explorative approach can be used to screen different sensors for appropriateness for different applications.

Sensitivity correction was based on introducing a reference standard solution in between every second sample measurement. The dynamic response curve of the reference solution is thus the evidence of sensitivity of the sensor array at the time of measurement. Significant variation between duplicate sensors on the array was eliminated as well as variation between replicate measurements of the same sample. Thus, in cases where the temporal drift of the sensor is significant or even unpredictable depending on the environment or the array itself there is no way around measuring a reference solution along with or immediately before or after measurement of the sample.

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