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On-line detection of microbial contaminations in animal cell reactor cultures using an electronic nose device

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Abstract

An electronic nose (EN) device was used to detect microbial and viral contaminations in a variety of animal cell culture systems. The emission of volatile components from the cultures accumulated in the bioreactor headspace, was sampled and subsequently analysed by the EN device. The EN, which was equipped with an array of 17 chemical gas sensors of varying selectivity towards the sampled volatile molecules, generated response patterns of up to 85 computed signals. Each 15 or 20 min a new gas sample was taken generating a new response pattern. A software evaluation tool visualised the data mainly by using principal component analysis. The EN was first used to detect microbial contaminations in a Chinese hamster ovary (CHO) cell line producing a recombinant human macrophage colony stimulating factor (rhM-CSF). The CHO cell culture was contaminated by *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida utilis* which all were detected. The response patterns from the CHO cell culture were compared with monoculture references of the microorganisms. Second, contaminations were studied in an Sf-9 insect cell culture producing another recombinant protein (VP2 protein). Contaminants were detected from *E. coli*, a filamentous fungus and a baculovirus. Third, contamination of a human cell line, HEK-293, infected with *E. coli* exhibited comparable results. Fourth, bacterial contaminations could also be detected in cultures of a MLV vector producer cell line. Based on the overall experiences in this study it is concluded that the EN method has in a number of cases the potential to be developed into a useful on-line contamination alarm in order to support safety and economical operation for industrial cultivation.

Introduction

Uncontrolled microbial and viral contaminations in large-scale animal cell culture systems are severe industrial problems. Examples of consequences of such contaminations are delay in production scheduling, inefficient use of expensive production facilities, and losses of high-value

biological products. Therefore it is essential to be able to sensitively and rapidly detect the contaminations in order to minimise these effects.

Currently applied methods for detection of biologically derived contaminations in cell culture bioreactors include unexpected shifts in dissolved oxygen (DO) tension, pH and the off-gas respiratory quotient. Although being direct, these methods are usually not very sensitive at the relatively low cell concentrations in cell culture production. Moreover, the shifts are difficult to observe when using on-line bioreactor control systems, since these compensate for changes in DO and pH. In most cases, this leads to long detection time for a contamination.

It has previously been shown that electronic nose (EN) devices (Kress-Rogers 1997; Dickinson et al. 1998; Gardner and Bartlett 1999) are useful for monitoring microbial and cellular activity in bioreactors by analyzing the effluent off-gas (Bachinger and Mandenius 2000). For example, the culture state of a Chinese hamster ovary (CHO) cell culture producing recombinant Factor VIII was monitored by an EN where also early detection of bacterial contaminations was demonstrated (Bachinger et al. 2000a, b, 2002). Furthermore, using similar ENs, strain identification methods for a number of microorganisms was shown in monoculture samples (Gibson et al. 1997; Gardner et al. 1998).

The EN devices use arrays of up to 50 chemical gas sensors with different but partially overlapping sensitivities to the volatile compounds in the sampled gas. Ideally, the spectrum of the responses represents a unique pattern, or fingerprint, of the composition of the analysed gases. Transient changes of gas composition result in characteristic variations of the shape of the pattern. By applying pattern recognition methods, for example partial least square regression (PLS) (Geladi and Kowalski 1986) or artificial neural networks (ANN) (Bishop 1995), compounds and their concentrations can be identified and estimated provided the recognition methods are correctly calibrated or trained.

EN devices are suitable for monitoring of bioreactor cultures due to several of their inherent properties. Both microbial and cellular cultures emit characteristic odours and these are often directly or indirectly related to the culture state. Thus, it is assumed that odorant emissions from the culture may under favourable situations reveal relevant information about the cells' conditions such as growth and productivity as well as occurrence of microbial contaminations. Also, the EN has the advantage of being non-invasive, contrary to pH and DO electrodes that normally must be immersed into the culture medium. Furthermore, the EN can easily be installed outside the sterile barrier of the bioreactor system, thereby allowing monitoring of the off-gas from several bioreactors at a production plant at the same time.

In this work the capacity of the EN for detecting microbial contaminations of a few currently used industrial animal cell cultures is assessed and compared. An EN device based on metal oxide semiconductor and field effect transistor sensors has been used. The requirements on the configuration of sensors in the EN arrays are described and the detection of several contaminations in cultures including CHO cells, insect cells, and human epithelial kidney (HEK) cells are demonstrated.

Materials and methods

Cell lines and cultivation media

A recombinant CHO cell line (ATCC CRL-10154) producing macrophage colony stimulating factor (M-CSF) was maintained in 250 ml spinner flasks at 37 °C under 10% CO_2 in air in an incubator. Ten millilitre of 10% Pluronic F-68 (Sigma), 20 ml of 200 mM L-glutamine (Invitrogen Life Technologies), 5 ml of penicillin streptomycin (10^4 IU/ml, 10^4 μg/ml, Invitrogen) and 1 ml of 1 mM methotrexate (Sigma) were added per litre medium. The CHO cell line was cultured in a serum free medium containing 3 g/l of glucose (SMIF-6, Invitrogen).

A *Spodoptera frugiperda* Sf-9 cell line (ECACC No. 89070101), previously adapted to SF900II serum-free medium (Gibco), was used (Cruz et al. 1997). Sf-9 was maintained in 250 ml (125 ml working volume) spinner flasks operated as repeated batch cultures at 27 °C and 170 rpm. Pluronic F-68 was added to the medium (2 g/l). For viral infections, a recombinant baculovirus, AcAs3-PPV (Casal 1996) was used. AcAs3-PPV was genetically modified to express the *LacZ* gene under the control of a *Drosophila* hsp70 promoter and to produce a *Porcine parvovirus* VP2 protein under the

control of the very late promoter p10.A human embryonic kidney cell line (HEK-293s), adapted to grow in suspension (Garnier et al. 1994; Côté et al. 1997), was kindly provided by Dr Amine Kamen (Animal Cell Technology and Downstream Processing Group, Biotechnology Research Institute, National Research Council Canada, Montreal, Canada). The HEK293s cells were cultured in a serum-free formulation consisting of a 1:1 mixture of FreeStyle 293 expression medium (Gibco-Invitrogen, catalogue No. 12338-026) and calcium-free DME medium. The NIH 3T3 based PG13 GFP (PG13-MND-GFP cl16) producer clone of retroviral vectors (GALV, transgene GFP)(reference for original cell line: Miller et al. (1991)) was maintained in a glucose-free DMEM (Axcell) supplemented with 20 g/l fructose and 5% FCS (Hyclone) in T-flasks at 37 °C under 5% CO_2 in air in an incubator.

Contaminants

Escherichia coli (DH5α PKS II β-gal), *Candida utilis* (CCUG 28186), *Pseudomonas aeruginosa* and *Staphylococcus aureus* (patient isolates, University Hospital, Linköping, Sweden) were cultivated in 100 ml SMIF-6 medium using 250 ml shake flasks. The flasks were inoculated with 1.5 ml suspensions of the strains (optical density (OD) 1.0–1.5). Cultivations were performed on a horizontal shaker at 37 °C. For the contamination experiments the microorganisms were grown overnight in 50 ml of SMIF-6 medium using 250 ml shake flasks, and a 1 ml suspension at an OD of 1.0 to 1.5 of the infectants were injected into the reactor.

For insect cell contamination experiments, *E. coli* (DH5α PKS II β-gal) was cultivated in 10 ml of LB medium, using a 100 ml shake flask, incubated overnight at 150 rpm in an orbital shaker at 37 °C. A second case study presented refers to an unintentional filamentous fungus contamination detected during a standard cell growth cultivation.

Viral infection

For the infection experiments, a recombinant baculovirus (AcAs3-PPV) was used for viral infections of Sf-9 insect cells to produce a *Porcine parvovirus* VP2 protein (Casal 1996). The infections were performed in the mid-exponential phase of cell growth ($3–4\times10^6$ cells/ml) with a multiplicity of infection (MOI) of 2–4 pfu/cell.

Bioreactor systems

CHO cell experiments were carried out in a 3-l bioreactor (Applikon, The Netherlands), temperature, DO and pH were controlled at 37 °C, 30% and 7.0, respectively, using an ADI 1030 Biocontroller. The working volume was 1.4 l. The same medium as in the spinner flasks was used, but without penicillin streptomycin. Cell density was determined under a microscope using a haemocytometer and trypan blue exclusion. Bacterial colony forming units (CFU) were counted on agar plates after serial dilutions of samples.

HEK-293 cells were cultivated in a bubble-free aerated membrane stirrer bioreactor at 37 °C, 12.5% CO_2 and 40–70 rpm (Wagner and Lehmann 1988). Dissolved oxygen was maintained at 40% of air saturation. The EN was connected to the off-gas line of the reactor. The bioreactor was inoculated at an initial cell concentration of about $3.0–5.0\times10^5$ cells/ml. For substance testing the membrane stirrer was replaced by bubble aeration to improve evaporation of the substances.

Insect cell experiments were performed in 2-l stirred bioreactors (B. Braun, Melsungen, Germany) using two six-blade Rushton turbines. Temperature was controlled at 37 °C and DO at 30% of air saturation varying the agitation rate and the oxygen concentration in gas inlet (a sparging mixture of nitrogen and oxygen, kept at a constant flow of 80 ml/min using mass flow controllers). Cultivations were inoculated at $3.0–5.0\times10^5$ cells/ml with cells derived from spinner flasks. Data acquisition and control were implemented in the Universal Bioprocess Control system (UBICON) (Electronic System Design, Hannover, Germany). The EN setup sampling was operated as above with the addition of a microfilter in the off-gas line.

The PG13 GFP cl16 cell experiments were carried out in a 2-l packed bed reactor system (Celligen from New Brunswick Scientific, the Netherlands), as previously described by Merten et al. (2001). The working volume was 1.4 l.

Temperature, pH, and pO_2 were controlled at the following values: 37 °C, 7.2, and 30% air saturation, the aeration was adjusted to supply a mixture of air, oxygen, nitrogen, and carbon dioxide at a flow rate of 100 ml/min. The same medium as in the T-flask cultures was used. The cell number per reactor could only be determined at the end of the culture by using a haemocytometer. Intermediate cell numbers were estimated by the daily glucose consumption. More details can be found in the paper by Merten et al. (2001). The EN was connected to the off-gas line of the reactor. The bioreactor was inoculated at an initial cell concentration of 60×10^6 cells/ml. The EN sampled the analyte gas from the bioreactor from its outlet of the off-gas cooler which was thermostated to 15 °C.

Electronic nose devices

The EN used in the experiments was especially designed for cell culture monitoring by AppliedSensor AB, Linköping, Sweden (Figure 1a and b) . It was equipped with 10 metal oxide semiconductor field effect transistors (MOSFET) sensors, five operating at 140 °C and five at 170 °C, one humidity sensor and six metal oxide sensors (MOS). Humidified, oil and particle filtered air was used as reference gas. The instrument was controlled by a software program designed for the EN (Senstool^{TM}, AppliedSensor AB). The software was also used for evaluation, data storage and data presentation (see below). Gas was sampled from the headspace of the culture vessel through a heated steel tube (37 °C). This was inserted into the bioreactor outlet connection, as described by Cimander et al. (2002) or through a piping system adapted to the bioreactor in use. Samples were collected every 15 or 20 min. Each gas sample acquisition consisted of 100 s of baseline determination, 20 s of sample and 1080 s of base line recovery. For each sample the response, on integral and derivative, and off-integral and derivative were calculated (see Figure 1c).

Data analysis

The initial and detailed data analysis was carried out by the custom designed data and signal evaluation program SensTool^{TM}. Sensor signals were computed by integration, derivation, subtraction and filtering functions were activated accordingly. Graphs in Figures 2 and 4 are results of these procedures. These data were further analysed by principal component analysis (PCA). PCA is based on a linear supervised pattern recognition technique that can reduce the dimensionality of multivariate data of the type the EN generate (Gardner and Bartlett 1999; Hines et al. 1999; Brereton 2003). The analysis algorithm results in two or more principal components, where the *n*th principal component describes the direction of the *n*th largest variation in the data set. The result is often presented in a so-called score plot where the two most important principal components are plotted against each other.

In this work the trajectory scores were calculated using a standard PCA-algorithm in the SensTool^{TM} software. The data sets from the EN responses were processes by the algorithm where normalisation methods according to basic statistical methods were used in the software. The PCA score plots presented have been computed by using standard PCA-algorithms. By adding a new score plot on an initially calculated plot it becomes possible to compare different cultivations. This is the case with the score plots presented in Figures 3, 5, 6 and 7.

Results and discussion

Measurement system

The EN response pattern from a cell culture may have various origins. The EN sensors can, if sensitive enough, respond (1) to specific volatile compounds emitted by the microorganisms or cells in the culture, or (2) to the emission from the nutrient components of the medium, either these are being metabolised by the organisms, remain in the medium or are chemically decomposed (Bachinger and Mandenius 2000). Furthermore, several of the parameters related to the design of the bioreactor and its operation may influence the volatility of low molecular-weight compounds, thereby impacting the EN pattern. However, most of these parameters are known and held constant during cultivation. If operational parameters are changed, for example agitation speed, this must be

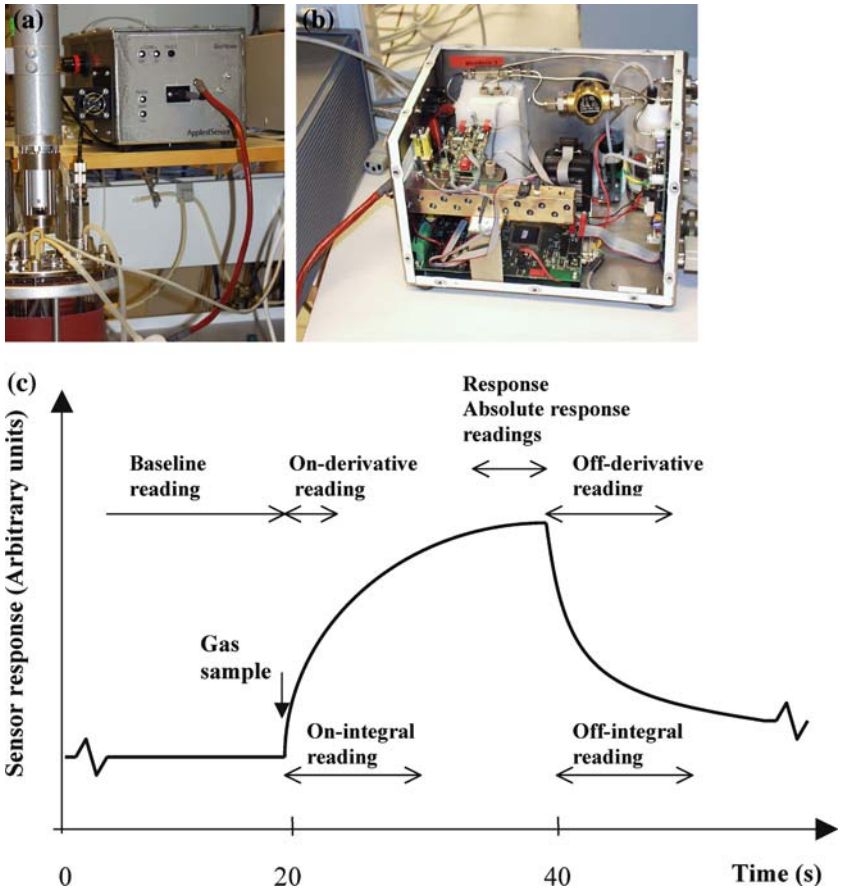

Figure 1. EN design and setup with typical response. (a) Electronic nose sensor unit monitoring a 2-l bioreactor, (b) picture of the setup, (c) response of one gas sample injection.

considered. Microbial or viral contaminants in the cell culture will behave accordingly; they will emit characteristic volatiles and may undergo changes due to growth, metabolism or decomposition, thereby changing the culture's emission as well.

In order to evaluate the sensitivity of the EN sensors to media or metabolic components that are likely to appear in cell cultures, a number of substances (Table 1) were dissolved in physiological buffer saline (PBS) at concentrations ranging from 10^{-8} to 10^{-2} M. As positive controls acetone and ethanol (diluted in PBS) were used since both are known to give high signals with the sensors in the EN. The solutions were filled in a cell culture bioreactor, thermostated at 37 °C and aerated with a typical gas mixture. The off-gas stream was analysed by the EN. As reference an identical bioreactor filled with pure PBS was used. Table 1 shows the responses obtained. The signals of the three highest responding sensors are given for each substance at 10^{-4} and 10^{-2} M concentrations relative to the signal at 10^{-8} M of the same substance. All tested substances, except acetic acid, showed significant but different response patterns. From these observations we assumed that signals caused by changes in the composition of culture media (e.g., nutrients like methionine) may interfere with responses correlated to substances that are secreted by animal cells or contaminating organisms. Both types of responses may be similarly useful for the detection of contaminations. Thus, the responses obtained in the experiments described in the following should be seen from this aspect and not necessarily be interpreted as caused by either the animal cell or the microbial contaminants.

The design and operation of the bioreactor were critical in several ways. The liquid volume/headspace

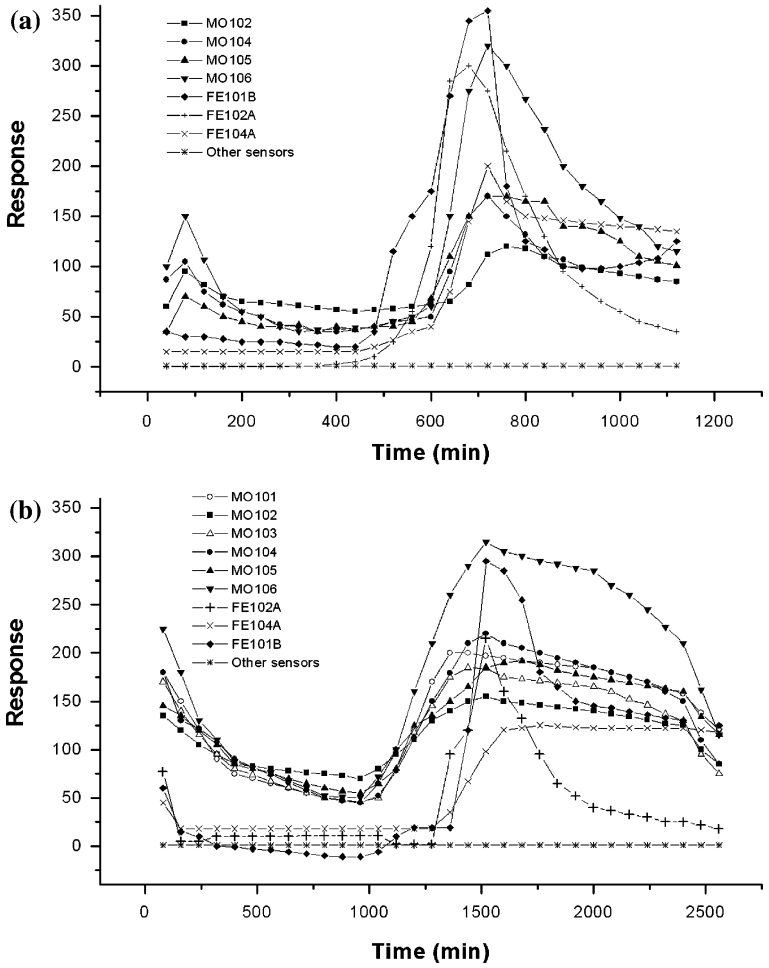

Figure 2. Trajectories for selected sensor signals, showing differences between shake flask cultures of (a) *E. coli*, (b) *C. utilis*, (c) *S. aureus*, and (d) *P. aeruginosa*. All sensor signals that were zero or very low are represented by one single graph (other sensors). Cell densities increased from 0.1 up to 2.5 (OD_{600}) during the cultivations.

ratio of the reactor affected the build-up of emission; something that had been taken into consideration especially at fed-batch operation. The temperature in the liquid, the headspace, and connections to the sensor array, influenced condensation of the gaseous analytes. To control the temperature as perfectly as possible in the whole analytical system from sampling point to detector was consequently necessary. Pressure drops, including head space overpressure if applied, also affected gas equilibria of analytes. Furthermore, the cooling of the bioreactor condenser influenced the amount of volatiles that reached the EN sensor array. Depending on specific requirements for insect cells, human cell lines, immobilised cells, different experimental setups and adjustments were made. For example, by using a condenser cooling flow of 15 °C the stability of the EN signals became better, although minor instabilities still interfered. When cultivating insect cells the optimal operation temperature in the culture was lower, thereby resulting in a lower volatility and concentration of the gaseous compounds in the off-gas. This could be compensated for by decreasing the flow rate of the carrier gas to the EN. This effect was also observed when cultivating human cells producing retroviral vectors (see below). In the case of the HEK and CHO cells a silicone tubing aeration system was applied for the reactor culture while the carrier gas for the EN was passed directly to the reactor headspace allowing sufficient amount of volatiles to be taken

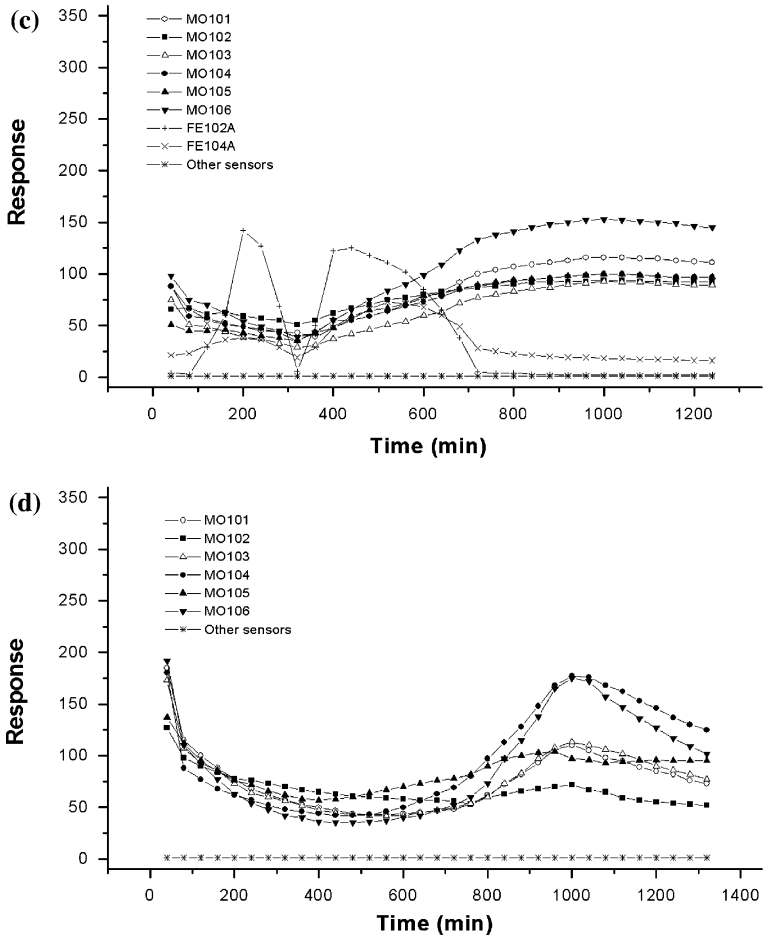

Figure 2. Continued.

up since the volatile emission from the culture apparently was sufficient. More details about the setups are given in the Material and methods Section above.

In particular, the aeration rate in animal cell cultures needed to be restricted since bubble sparging can be lethally damaging to the cells (Oh et al. 1992). Other studies on stresses associated with bursting bubbles (Boulton-Stone and Blake 1993) showed that they were much greater than those due to fluid turbulence (Zhang and Thomas 1993), the latter being insufficient to rupture the cells. In this context, it could also be shown that the use of Pluronic F68 protects cells from lethal damage because, amongst others, the surfactant prevents cells attaching to the bubbles, thereby keeping them away from the regions of highest stress during bubble bursting (Chalmers and Bavarian 1992).

With lower aeration rate the interface area and, in particular, the bursting of the bubbles are reduced and with this the shear stress on the cells. Aeration also leads to ample foaming in serum containing media. These effects have to be considered, and, if possible minimised when sampling for EN analysis. An important observation was that a decrease of the aeration rate from 150 to 35 ml/min at 25% of the culture time (use of an on–off mode) in a 1.4-l culture of a Phoenix A cell line producing a recombinant MLV-GFP (Green fluorescence protein) resulted in 8- to 9-fold increase in sensitivity of the EN signal (not shown). Theoretically, this correlated well with the expected accumulation of volatile substances.

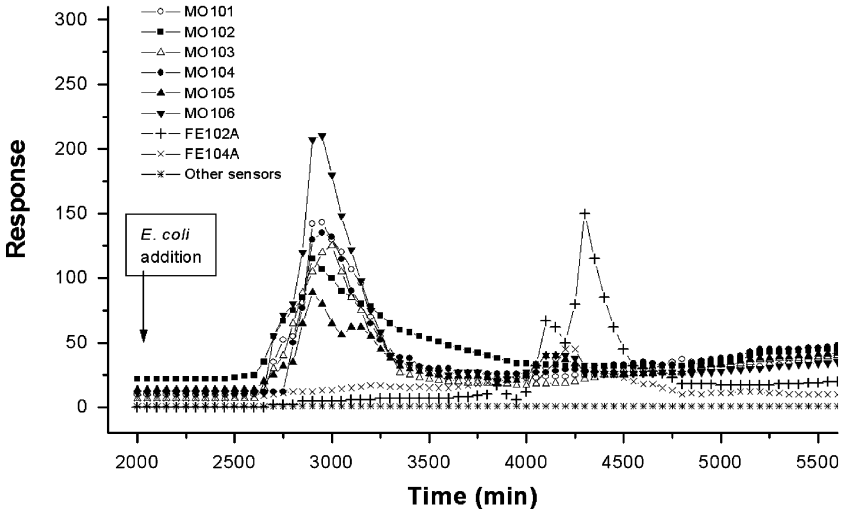

Figure 3. Trajectories for a few selected sensors during a reactor culture of CHO cells infected with *E. coli* (addition at the arrow of 1 ml of 1.6×10^9 cells/ml).

CHO cell culture contamination

Four contaminating microorganisms common in industrial CHO cell cultures were examined. Distinctly different EN response patterns were obtained for each of the strains when they were grown in independent shake flask monocultures in a serum-free CHO cell medium (Figure 2). As discussed above, the medium itself gave rise to a background of responses caused by the emission from its components. When the cell density of the infecting microorganism became sufficiently high it generated characteristic strain-specific response patterns in all monocultures (Figure 2a–d). As evident from the graphs a limited number of the sensors were the main contributors to the general shape of the response patterns. For example, the MOSFET-sensor FE102A responded significantly in the *E. coli*, *C. utilis* and *S. aureus* monocultures (Figure 2a–c), but not in the *P. aeruginosa* culture (Figure 2d). The MOS-sensor MO101 gave high responses in the *C. utilis*, *S. aureus* and *P. aeruginosa* monocultures (Figure 2b–d) but not in the *E. coli* monoculture (Figure 2a). Furthermore, the dynamics of the sensor responses were different (i.e., rise and fall times of the signals), which partly may be explained by variations in growth kinetics of the microorganisms, partly by their intrinsic metabolism.

Contaminating amounts of the microorganisms were subsequently transferred to a 2-l bioreactor with a CHO cell culture growing in the same medium as the monocultures (1 ml of suspensions were added with concentrations of 1.6×10^9 cells/ml for *E. coli*; 2.8×10^9 cells/ml for *S. aureus*; 5.5×10^9 cells/ml for *P. aeruginosa*; 1.5×10^7 cells/ml for *C. utilis*). The CHO cells had been under

Table 1. Responses to solutions of potential cell culture components in PBS at different concentrations.

Component	Main responding sensors	Signal (response) at[a]	
		10^{-4} M	10^{-2} M
Acetic acid	FE104B	0.09	−0.68
	FE101B	0.03	−0.09
	FE102B	0.01	0.14
Ammonium chloride	FE105B	−0.31	47.58
	FE103B	−1.78	45.98
	FE101B	−0.28	47.62
Lactate	MO104	0.26	29.02
	MO101	0.05	25.62
	MO102	0.23	19.03
Methionine	FE102B	118.84	192.68
	FE104B	79.81	92.18
	FE101B	16.52	90.61
Acetone	MO106	67.58	236.16
	MO104	65.00	196.94
	MO101	52.39	110.47
Ethanol	MO106	6.47	244.43
	MO104	12.43	206.50
	MO101	9.18	135.98

[a]As a ratio versus the response at a concentration of 10^{-8} M of the component.

exponential growth for 24–36 h before the transfer. The initial medium contained such quantities of nutrients that it could sustain further growth of both the CHO cells and the microbial contaminant. As in the shake flask cultures, the medium with CHO cells gave prior to transfer of the contaminant a characteristic response. Once the contaminant reached a certain level in the culture the EN signal increased sharply, thereby exhibiting a response pattern aberrant from the normal CHO cell pattern.

When adding *E. coli* to the same amount as above, this response increase extended over 750–1000 min, but was later followed by a second increase in one sensor (FE102A) approx. 1000 min later (Figure 3). The first increase was assumed to be an effect of *E. coli* growth on the SMIF-6 medium while the second a result of growth on components excreted to the medium from lysed CHO cells. This biphasic behaviour was only observed with *E. coli* contamination. Figure 3 also reveals that a shift occurred in the ratio of response amplitudes, i.e., signal heights, between the sensors compared to the shake flask cultures. Similar shifts occurred when *C. utilis*, *S. aureus* or *P. aeruginosa* contaminated the CHO cell cultures (data not shown). In addition to the interactive effects observed in the mixed cultures of CHO cells and contaminating microorganisms the different operational conditions of the bioreactor compared to the shake flasks influenced the response patterns. Also, the CHO cells might have modified the growth medium leading to a change in metabolism of the contaminating *E. coli*, and thus to shifts in sensor responses.

The complexity of the sensor pattern motivated a detailed principal component analysis (PCA) (Brereton 2003). In order to generate principal component (PC) trajectories the response data were computed by a standard PCA-algorithm (Wold 1987; Gardner and Bartlett 1999). The anomalies observed in the response patterns were more clearly mirrored in the PC trajectories (Figure 4). The score plot representations of the trajectories are here shown by two PCs. As typical in PCA (Hines et al. 1999) the main portion of the variance is in the first PC while the second and higher PCs contain a gradually decreasing portion of the variance. In most of the cases, the PC1 and PC2 together covered 94.0–99.0% of the variance. As can be noted, the scalings of the axes are quite different; the *C. utilis* plot exhibits significantly smaller values (Figure 4b) than for example the *E. coli* plot (Figure 4a) and the non-contaminated culture (Figure 4e). The general pattern of the PCA plots was possible to repeat, although variations in individual sensors gave rise to deviations. This is explained by the fact that different scaling was used on the data at the PCA. The obtained responses were reproduced in repeated CHO cultivations where each contaminating strain produced characteristically distinctive PC patterns versus uninfected cultivations.

Microbial contamination of insect cell cultures

Also when contaminating an insect cell culture with microbial contaminants deviating response patterns were obtained from the EN. To evaluate the ability of the EN to follow up microbial contamination, two set of experiments were performed, under the same bioreaction conditions: Sf-9 insect cell growth without contamination, and Sf-9 cultures contaminated by an *E. coli* strain in a lab-scale bioreactor system (for setup details, see Material and methods Section). The off-gas sampling system of the EN setup was, due to the specific requirements of insect cell lines, altered whereby basic responses from the EN sensors produced as expected patterns of new shapes, as explained above; thus, the basic PCA trajectory score plot exhibited other shapes than was obtained with the CHO cell culture above. Figure 5 shows the PCA profiles of 4 independent bioreactions (BN2, BN3, BN4 and BN5) for Sf-9 cell growth cultures. Reproducible, and almost overlapping patterns were obtained, allowing this set of experiments to be used as a Sf-9 cell growth model. The contamination by *E. coli* did show a quite different pattern, and a strong deviation from the normally growth of the uncontaminated insect cell culture was exhibited (Figure 6).

A clearly different deviating profile was obtained as well when a fungus contaminated the same insect cell culture (Figure 7a). Contaminations with fungi occurring in insect cell cultures are quite common and difficult to detect by common methods especially at early cultivation stages, as visual microscopy, monitoring DO or pH signals or the rheological properties of the culture often remains similar to those from standard

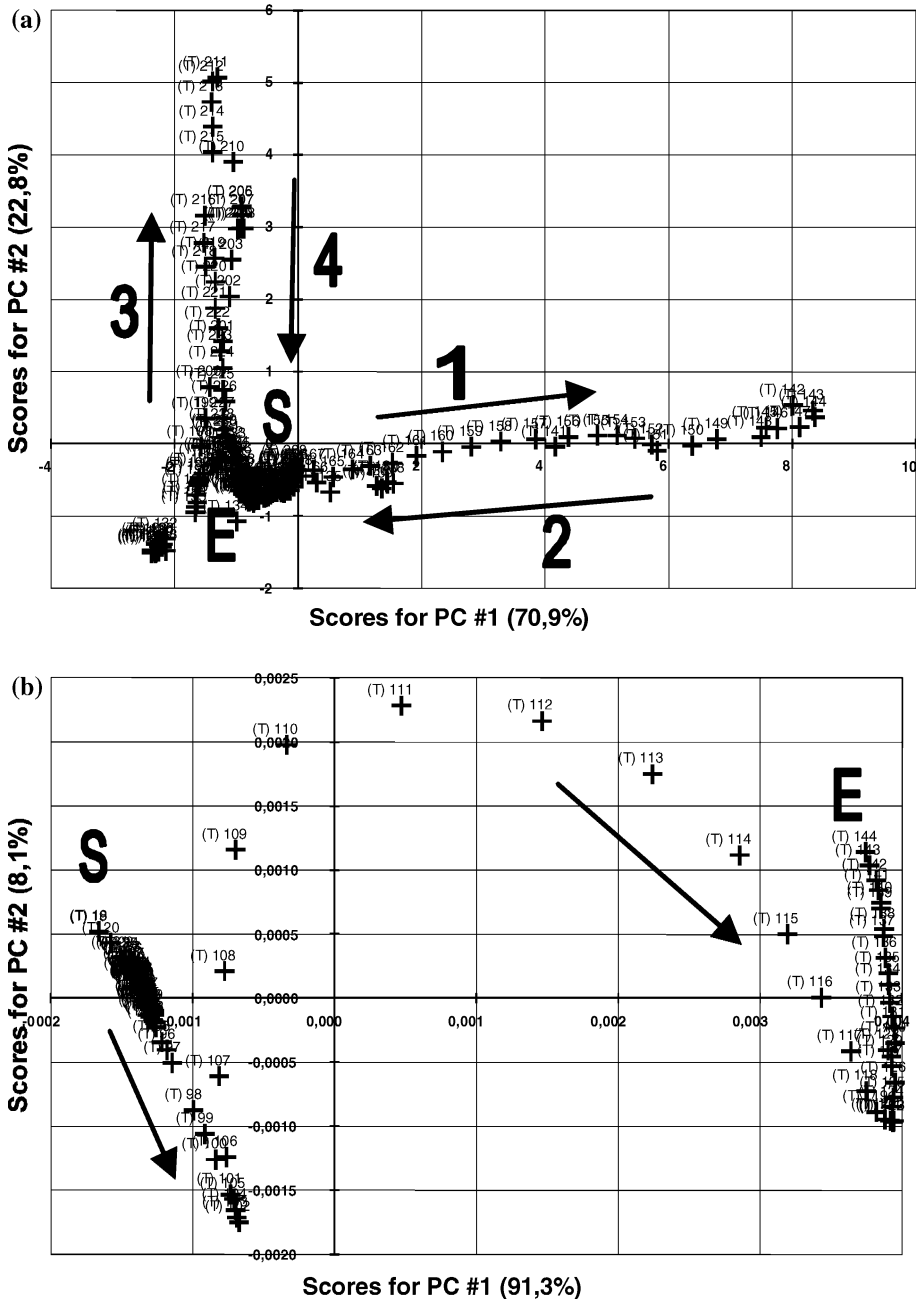

Figure 4. PCA score plots for CHO cell cultures contaminated by (a) *E. coli*, (b) *C. utilis*, (c) *S. aureus* and (d) *P. aeruginosa*, and (e) a non-contaminated culture. S, start of trajectory; E, end of trajectory. CHO cell count increased from 0.10×10^6 cells/ml up to 1.8×10^6 cells/ml during the cultivations. In all of the PCA plots it is possible to see how the cultures progress from start to end. The CHO cultures contaminated with microorganisms (a–d) exhibit clearly different trajectories and these are all different from the trajectory of a non-contaminated CHO-cell culture (e). The PCA plots indicate the possibility to use the electronic nose for tracking the contaminating events.

cultivations. However, when plotting EN data from the contaminated culture (BN10) onto the PCA model built from previously sampled culture data (bioreactions BN2 to BN5) a clear deviation from the established growth profile was obtained, thereby allowing the contamination detection: a

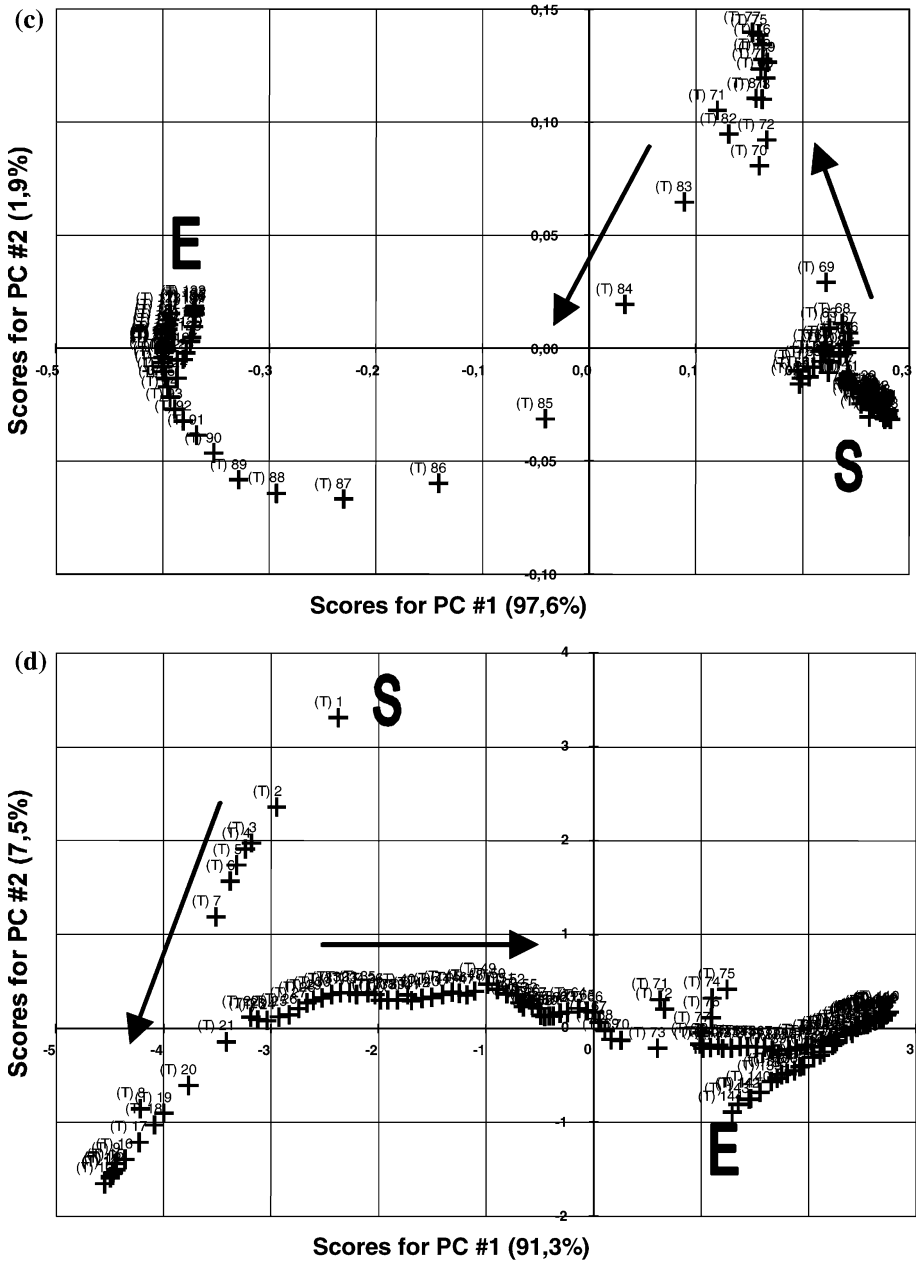

Figure 4. Continued.

slight but steady deviation of the contaminated culture PCA trajectory could be observed after approximately 115 h of cultivation (Figure 7a). Detection of this contamination at this stage was not possible by analysing the on-line acquired data of pH and oxygen flow profiles as shown in Figure 7b, where no differences can be observed between the contaminated cultivation (BN10) and the standard growth cultivations (BN2 to BN5).

Viral infection of insect cell cultures

Infection of insect cell cultures is a standard method for the production of recombinant proteins,

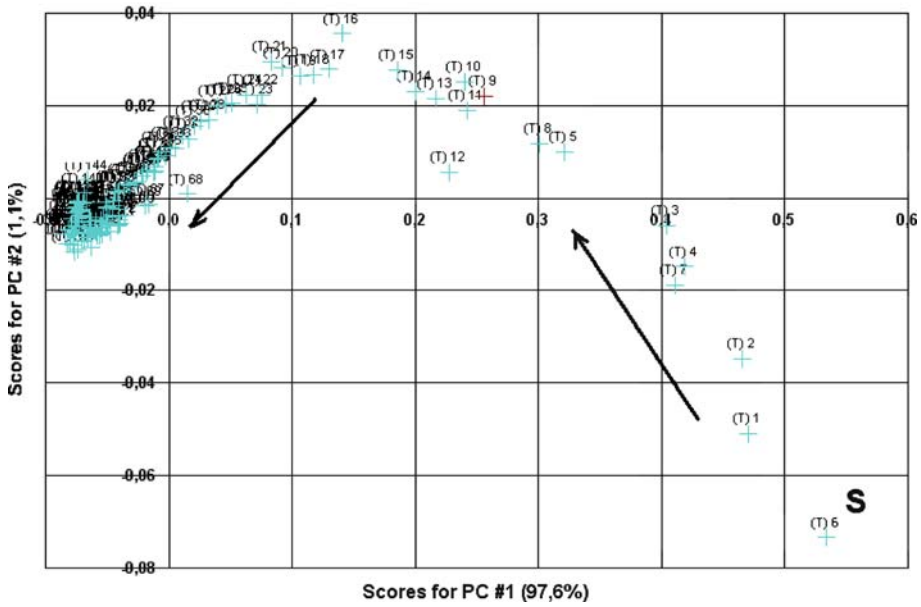

Figure 4. Continued.

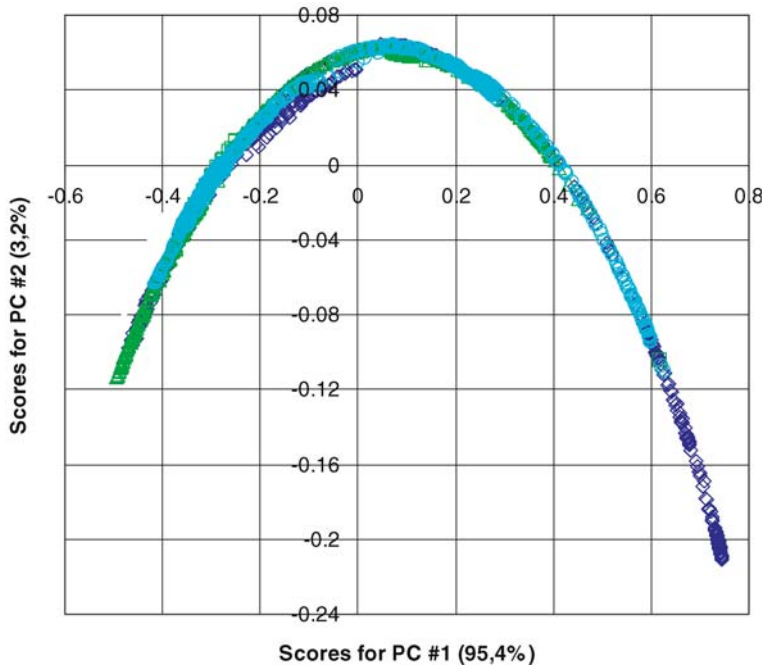

Figure 5. Sf-9 cell growth culture model. PCA score plot for four non-contaminated cultures: BN2 (◇), BN3 (□), BN4 (△) and BN5 (○). The similarity of the four trajectories indicate that insect cell cultures which are not contaminated exhibit almost the same shape when cultured repeatedly. Obviously the electronic nose is in this case capable of accurately reproducing the sensor responses from a non-contaminated culture.

virus like particles (VLP's) and viral vectors. After establishing the PCA model for cell growth Sf-9 cultures as described previously, experiments were performed with baculovirus infection for PPV-VLP production. The insect cultures were infected in the middle of its exponential phase at a cell

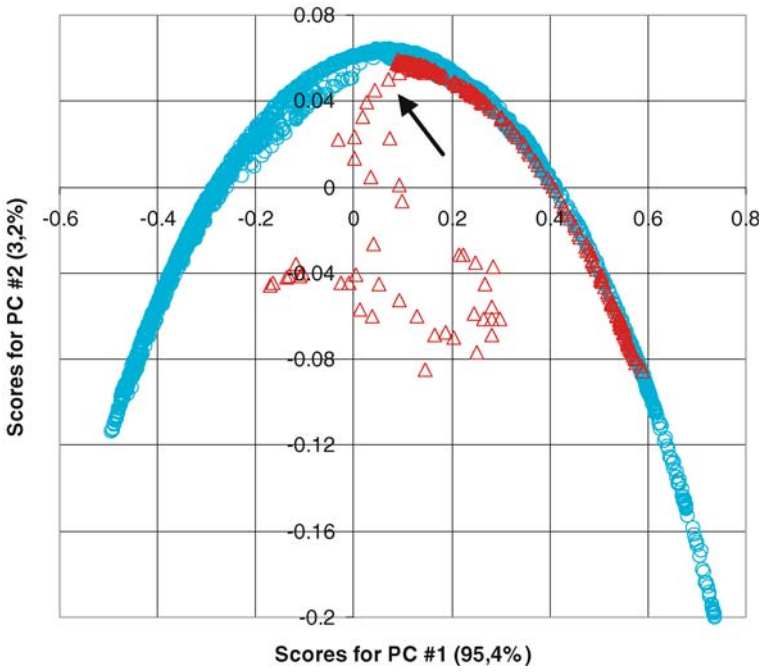

Figure 6. Contamination of an Sf-9 cell culture by *E. coli*. PCA plot for contamination (BN11) (△), and for contamination-free cultures (BN2 to BN5 overlapping data) (○). Arrow indicates a clear deviation from cell growth profile. The trajectory from the *E. coli* contaminated insect culture significantly deviates from the non-contaminated cultures almost directly after the addition.

concentration of 4×10^6 cells/ml. Two levels of multiplicity of infection (MOI) were applied (MOI of 3.76 and 2.33 pfu/cell). The PCA profiles obtained in these experiments (Figure 8) show the same trajectory as the reference cultivations (Figure 5) until the baculovirus addition. After infection, the PCA trajectory stops around the same PC#1 and PC#2 coordinates, revealing that the cells were successfully infected.

To study the EN response to the infection kinetics, a different kind of data analysis was performed by scaling the data to unit variance in the PCA trajectories. When applying this scaling to three similar batches (two uninfected cultivations, BN17 and BN19 and one cultivation batch infected by baculovirus, BN16) it became possible to reveal the infection process. During the first 14 h post-infection, the PCA trajectory remained similar to the ones obtained for cell growth; subsequently the PCA trajectory suddenly deviated from the typical growth profile, and entered a different shape which was defined as the production stage region of the PCA score plot (Figure 9a). Moreover, when plotting the two principal components (#1 and #2) in a score to time diagram together with the specific oxygen consumption rate time profile during the critical period, it can be seen that the transition point observed in the PCA trajectory at 14 hpi corresponds to the maximum specific oxygen consumption rate of the culture (Figure 9b). Thus, this important variable can directly be easily detected in a PCA score plot.

Further examples for the detection of contaminations in other mammalian cell cultures

Detection of contamination of a human cell line (HEK293) producing a recombinant protein by *E. coli* was possible as well. The results of these experiments did not show any principally different patterns than shown above from CHO and insect cell culture experiments. Since the *E. coli* infection had characteristic responses in the EN patterns, it is expected that other cell lines in a serum-free cultivation medium permits rapid detection as well.

Bacterial contaminations (unidentified bacteria) were observed in two reactor cultures performed

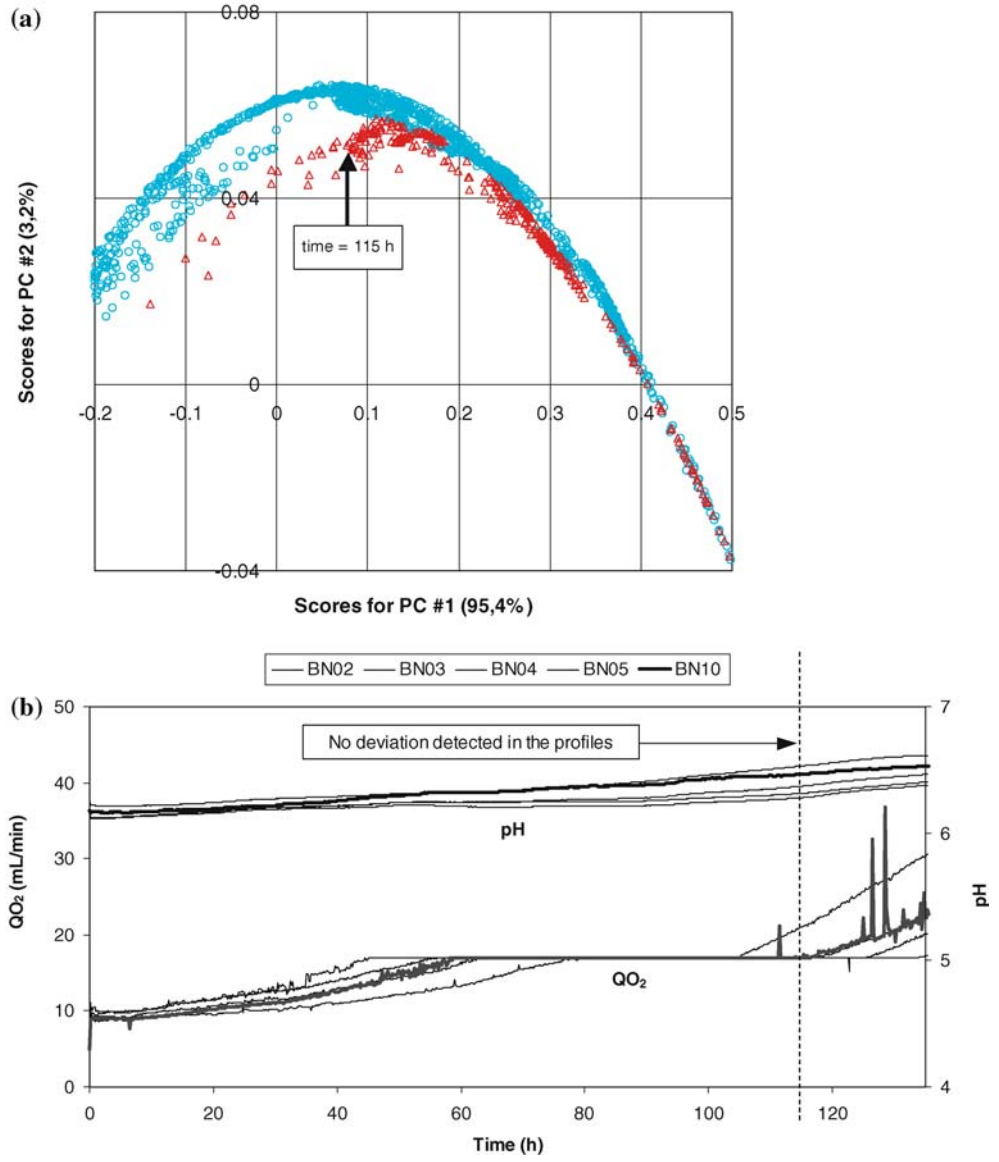

Figure 7. Contamination of an Sf-9 cell culture by a fungus. (a) PCA plot for contamination (BN10) (△), and for contamination-free cultures (BN2 to BN5) (○). Arrow indicates a clear deviation from cell growth profile, corresponding to detection of the contamination at around 115 h of culture time. (b) Profiles of on-line data acquisition of pH and oxygen flow for contaminated culture (BN10) (thick lines), and for contamination-free cultures (BN2 to BN5) (thin lines). The trajectory from the contaminated culture does in PCA score plot deviate more significantly than the pH and QO_2 signals do in the time plot at 115 h. This indicates that the electronic nose has a better capability for early detection of the contamination.

with PG13 GFP cells. Comparing a non-contaminated reference culture with a culture contaminated at the onset of this culture indicated very clearly that the MOS sensors (Figure 10, shown for the MOS104 sensor, however, completely equal behaviour observed for the five other MOS sensors of the EN) were able to clearly distinguish between a non-contaminated and a contaminated culture. In the case of the uncontaminated reference culture, the sensor output started to significantly decrease from 48 h post-inoculation onwards, which correlated rather well with the cell growth (inversed relationship), and can be explained by either depletion or masking of one or several medium components by the cell growth. On the other side, for the contaminated culture, the sensor

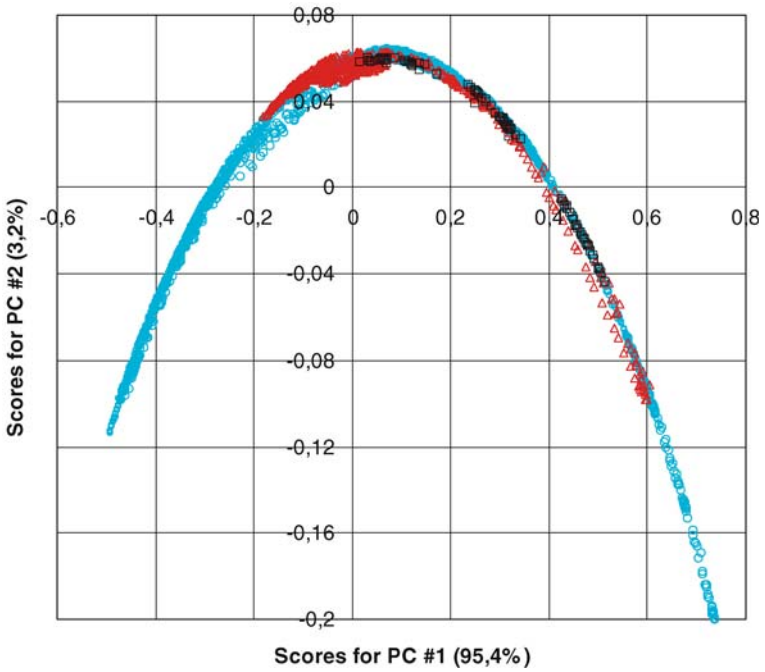

Figure 8. Infection of an Sf-9 cell culture by a baculovirus. Plots for cultivation with infection at MOI = 3.76 (□) and at MOI = 2.33 (△) and for infection-free cultures (BN2 to BN5) (○). At the infection instant (at the maximum of the plots) the trajectories separate but subsequently coincide again when the baculovirus is taken up by the cells.

signal stayed constant for the first 48 h of the culture and then started to increase in connection with the development of the bacterial contamination. This significant increase in the sensor signal is probably elicited by the production of volatile compound(s) by the contaminating microorganisms.

Although this type of sensor is able to detect a bacterial contamination very clearly (due to an important modification of the gas composition of the exit gas) it should be mentioned here that the occurrence of a visible contamination preceded its detection by the MOS sensors (by about 10–15 h), which depends on different reactor parameters (max. aeration rate, amount of oxygen in the gas mix, mass transfer performance of the reactor, etc.) and is an issue of sensor sensitivity. However, it is also possible that the delayed response of the sensor is due to a delayed production of the volatile substances due to an association of their production with a certain physiological state. If this is the case, the bionose might only be of limited use for the early detection of microbial contaminations.

Conclusion

In this study we have investigated the possibilities of detecting microbial and viral contaminations in a number of currently used industrial animal cell lines by using an electronic nose device. The objective was to find a better means to more rapidly and accurately determine the contaminations in order to be able to counteract those economical and practical problems they cause in industrial development and production work, in particular when producing pharmaceutical products such as recombinant proteins, viral vectors and monoclonal antibodies.

In the case of microbial contaminations of a commonly used CHO cell line producing a recombinant protein it was shown that the EN can distinguish between several typical microbial contaminants. In the case of the often used insect cell line Sf-9 contaminations of two bacteria, a fungus, and a virus were detected with good results.

In those cases where the EN responded rapidly and accurately the device provides a tool with interesting potential for industrial bioprocess monitoring and control routines in order to increase

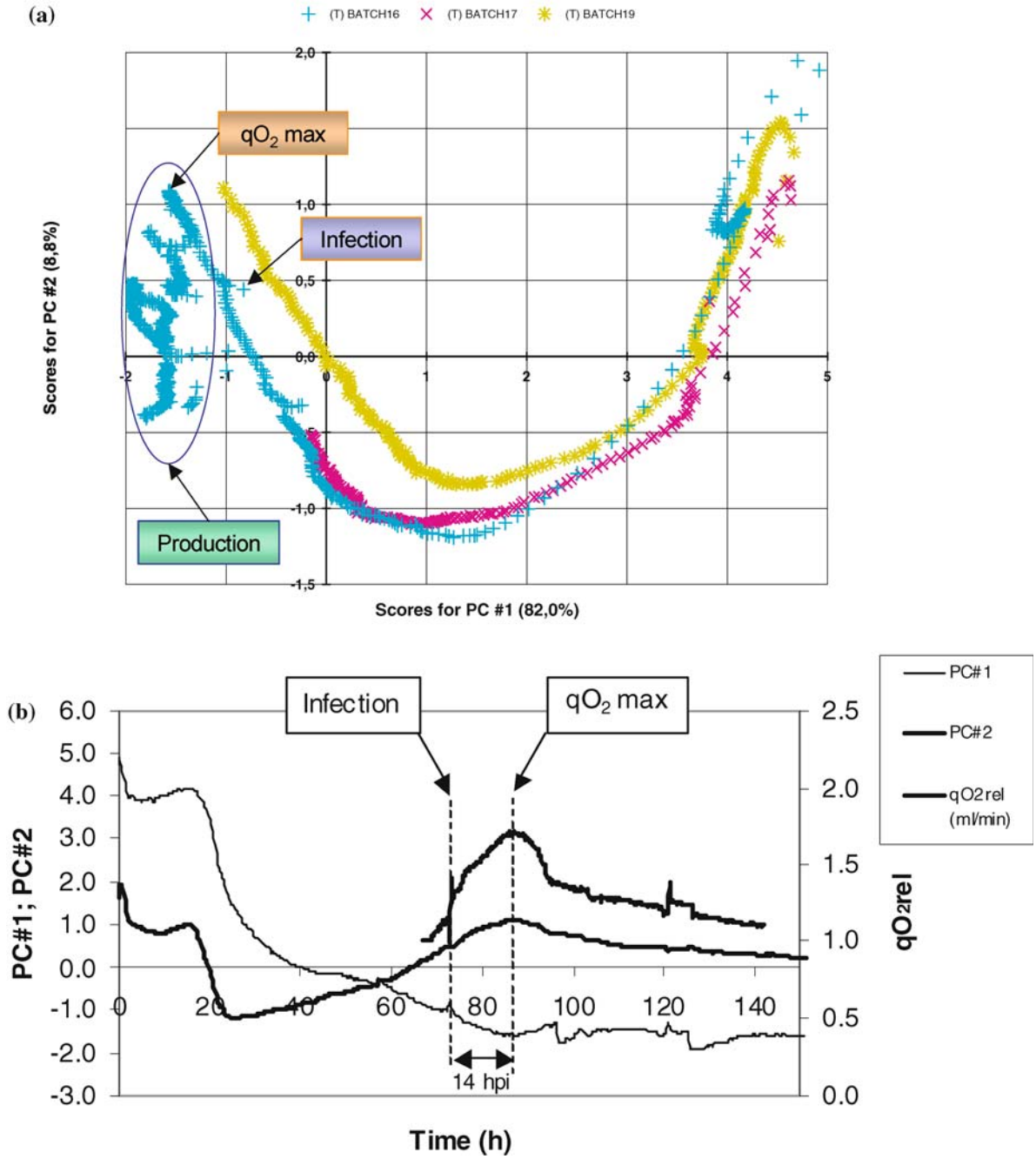

Figure 9. Study of the course of the virus infection process. (a) PCA score plot using unit variance scaling for an Sf-9 cell culture infected by baculovirus (BN16) (+) and for two infection-free cultures (BN17 (×) and BN19 (∗)). The instances and period of infection, production stage and maximum specific oxygen consumption qO_{2max} are indicated. (b) Time graphics of the two principal components in (a) over time for the BN16 culture. The correspondence between the production transition point for the culture as detected in the PCA plot (a), being 14 h post-infection (hpi), and the occurrence of maximum specific oxygen consumption as monitored in the time plot (b) can here be compared. The electronic nose data indicate that they have the potential to better visualise the course of the virus infection process in insect cell cultures.

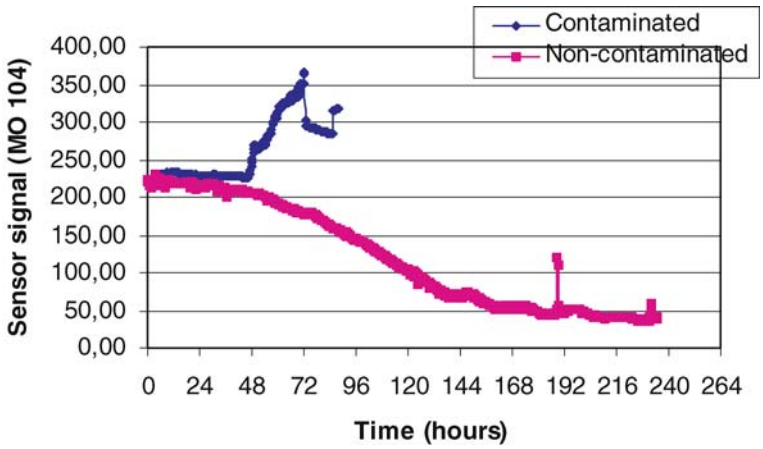

Figure 10. Sensor response of the MOS 104 sensor in a non-contaminated (square) and a contaminated (diamond) reactor culture of PG13 GFP. Both cultures have been inoculated with 10^7 cells/ml: at the end of the non-contaminated culture (at 234 h), 5.2×10^9 cells/ml were counted. The sensor signal values of the contaminated culture have been normalised with respect to the reference culture at the culture onset time (0 h).

safety and minimise economical losses. An advantage is its non-invasive sampling procedure which allows connection to any cultivation system without interfering with the culture. Also, the EN provides a detection system that in a number of combinations of cell lines, media and gas sensors, has the potential to detect contaminations more sensitively than, for example pH and DO electrodes as previously shown for bacteria (Bachinger et al. 2002). However, this necessitates that stable EN sensors are at hand that respond to one, or better, a few characteristic compound(s) that are not more easily characterised by bioreactor electrodes.

It should also be emphasised that successful application of the EN devices depends on a precise and careful installation of the gas sampling system in order to adjust and compensate for effects related to condensation of volatile compounds, sample transformation, the composition of medium components, intrinsic properties of the cell line, and occurrence of components in the gas that may cause slow reversible or irreversible sensor memory effects. These effects may be negated by applying specific sensors, cooling devices, gas filters, carrier gas flow, or other means for compensation. However, all of these measures require additional verification tests for the particular cell production system in use.

In order to more completely appreciate the usefulness of the EN the list of contaminants needs to be extended with a number of other commonly occurring contaminating species in an industrial environment, such as mycoplasmas or non-lytic, integrating viruses. In addition, the possibility of using other chemical gas sensors in the EN sensor array that are complementary to other contaminants is recommended to be further investigated.

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