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A new respirometric endpoint-based biosensor to assess the relative toxicity of chemicals on immobilized human cells

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ABSTRACT

Several functional and biochemical parameters have been proposed as biomarkers of effect of environmental pollutants. A rapid biosensor working with immobilized human U-937 cells was developed and applied to environmentally relevant chemicals with different structures and toxicological pathways, i.e. benzalkonium chloride, clofibric acid, diclofenac, mercury nitrate, ofloxacin, and sodium dodecyl sulphate. Respiration of cells was relied upon as a comprehensive biochemical effect for screening purposes. Analytical parameter ($\Delta ppmO_2$) and toxicological index (respiratory inhibition, $\delta\%$) measured after 1 h of exposure were utilized for dose–response relationship study. Results (toxicity rating scales based on $\delta_{50}\%$ and steepness) were compared with those obtained by the same approach previously optimized on *Saccharomyces cerevisiae*. The toxicity rating scale obtained by the biomarker based on human mitochondrial and cell metabolic activities compared well with previous scale obtained on yeast cells and with available *in-vivo* acute toxicity indexes; respiration was confirmed as toxicological endpoint reliably measurable by the biosensor.

1. Introduction

The development of novel, sensitive and efficient *in-vitro* bioassays and strategies is recommended in order to monitor human exposure to a broad-range of chemical agents as well as rapidly determine potential implications of these exposures to human health (Spielmann et al., 2006). Biosensors have many advantages, such as simple and low-cost instrumentation, fast response times, and high sample throughput. A biosensor is an analytical device consisting of a chemical sensor and a biologically active detection system: the reaction of the biological system with the analyte produces a change in the parameter which, in its turn, is measured by an appropriate transducer (Hictchman, 1978). To identify threshold values for exposure through foods and/or environment via dose–response studies, biosensors based on yeast cells has been developed and widely applied (Haubenstricker et al., 1990; Campanella et al., 1995, 1996, 2000; Hollis et al., 2000; Frazzoli et al., 2007); biosensors based on mammalian cells have been also developed (Riley et al., 2003; Chin and Kwun, 2007). Object of this study was the development of a respirometric biosensor based on human cells to further reduce uncertainty factors in the assessment of toxicity on humans. The proven wide field of applicability of the respirometric biosensor on yeast model (Campanella et al., 1995, 1996, 2000; Frazzoli et al., 2007) suggests respiration as reliable endpoint for application also on human cells. In fact, cellular respiratory chain, as well as the associated electron transport in mitochondria, are targeted by several groups of chemicals, e.g. a number of different pesticides (Leroux and Delorme, 1997), heavy metals (Gustavson et al., 2002; Strydom et al., 2006; Frazzoli et al., 2007), drugs (Ishikawa et al., 2006; Tao et al., 2006; Scatena et al., 2007), and metabolites (Weinbach and Ebert, 1985). Cellular respiratory rates are normally determined by metabolic activity, covering more than 20 enzymes. Respiration has been studied to determine whether any change in oxygen consumption occurs under optimal nutritional conditions and normal O_2 tension (155–159 Torr, at barometric pressure of 760 Torr). The Henry's law ($p_a = \chi_a k_a$) describes the *equilibrium* between the O_2 partial pressure (p_a) and the dissolved O_2 molar fraction (χ_a). When temperature is set, the k_a value is constant. Physical–chemical properties of oxygen allow its diffusion through the lipid layers of the cellular membrane. Respiration may be inhibited by the exposure to chemicals able to affect cell membrane, mitochondrial functions key points in the metabolic chain.

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In the present study, we investigated the ability of a set of chemicals with different structures (Table 1) to inhibit respiration of human cells, through the O_2 measurement during the metabolism of a relevant nutrient (glucose) in open system (at 37 °C). The dose–response curves were constructed to determine the slope (potency of the toxicant) and the 50% inhibition of respiration value (δ_{50}).

The biosensor was assembled by coupling a Clark-type O-sensing electrode (used as amperometric transducer of the cellular respiratory activity) to the biological medium, i.e. a disk containing cells immobilized by inclusion in agarose. A protocol was developed to properly prepare the biological media; in particular, human myelomonocytic U-937 cell line derived from a patient with histocytic lymphoma (Sundstrom and Nilsson, 1976) was used. This cell line was chosen because it grows in suspension medium and trypsinization, which can stress the cells and possibly interfere with respiration, is avoided. Moreover, large amounts of cells can be easily obtained by suspension culture. Chemicals selected for investigation included environmentally relevant and widely used substances: clofibric acid, diclofenac, and ofloxacin were selected as active pharmaceutical ingredients (APIs) able to bioconcentrate in hospital effluents, as well as in urban sewage sludge (Zuccato et al., 2000; Ferrari et al., 2004). As regards contaminants, physical, chemical, and biological properties of heavy metals allow them to persist in the body and cause chronic effects; among heavy metals, mercury represents an "hot" metal and its double charged ion (e.g. $Hg(NO_3)_2$) presents good aptitude to bind S atoms in proteins and interfere with essential enzymatic activity (ATSDR, 1999). Further, the exposure to widely and increasingly used surfactants with different mechanisms (Grant and Acosta, 1996) was considered, and both cationic (benzalkonium chloride) and anionic (sodium dodecyl sulphate) agents were assayed.

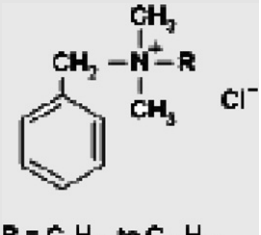
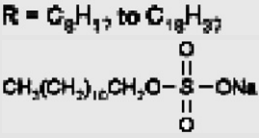
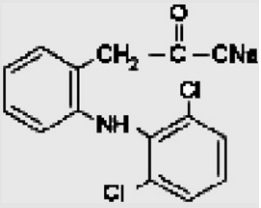
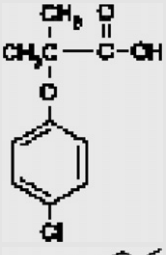
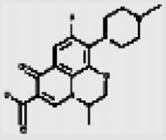
As mentioned, results were compared with those previously obtained on *Saccharomyces cerevisiae* yeast cells and information on the acute toxicity on laboratory animals, e.g. LD_{50} values (median lethal dose), was derived from Material Safety Data Sheet (MSDSs) on these compounds.

2. Materials and methods

2.1. Reagents

Diclofenac sodium salt (CAS no. 15307-79-6, Sigma-Aldrich) is the active principle used in a variety of drugs, such as Dealgic, Deflamat, Diclocular, Diclofan, Diclofenac Hexan, Dicloftil, Dicloreum, Dolaut, Fenadol, Flogofenac, Forgenac, and

Table 1
LD_{50} values, formula, MW, and structure of the chemicals under study, as obtained from the MSDSs

Chemical	LD_{50} (mg/kg)	Formula	MW (g/mol)	Structure
Mercury nitrate monohydrate	ORL-RAT 26	$Hg(NO_3)_2 \cdot H_2O$	342.62	
Benzalkonium chloride	ORL-RAT 240	$C_{24}H_{42}NCl$	350.65	
Sodium dodecyl sulphate	ORL-RAT 1300	$C_{12}H_{25}NaO_4S$	288.38	
Diclofenac	ORL-RAT 53	$C_{14}H_{10}Cl_2NO_2Na$	318.13	
Clofibric acid	ORL-RAT 897	$C_{10}H_{11}ClO_3$	214.65	
Ofloxacin	ORL-RAT 3500 (Exocin)	$C_{18}H_{20}FN_3O_4$	361.37	

Voltaren. It has analgesic and anti-inflammatory properties correlated to the peripheral inhibition of prostaglandin synthesis. Ofloxacin (CAS no. 82419-36-1, Sigma-Aldrich) is the synthetic active principle used in Exocin, Flobacin, and Oflocin, and presents a wide antibacterial range of activity. As regards clofibric acid (CAS no. 882-09-7, Sigma-Aldrich), it represents the main metabolite of clofibrate, which in its turn is an antiperlipidemic drug with high environmental persistence (estimated as ca. 21 years).

Mercury nitrate monohydrate (CAS no. 7783-34-8), sodium dodecyl sulphate (CAS no. 151-21-3), benzalkonium chloride (CAS no. 8001-54-5, Fluka), formaldehyde (CAS no. 50-00-0), and D-(+)-glucose monohydrate (CAS no.14431-43-7) were obtained from Fluka. Standards solutions of chemicals were prepared through proper dilution with high purity deionized water from Millipore Milli-Q system. The LD_{50} values of the selected chemicals are reported in Table 1, along with other information of interest.

2.2. Cell culture

Human leukaemia U-937 cell line (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 (GIBCO) medium containing 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 i.u./ml penicillin, 50 μg/ml streptomycin (GIBCO), and 20 mM HEPES buffer (Sigma). Cells were cultured in T-75 tissue culture flasks (Corning, Corning, NY), at 37 °C. Every 4 days cells were collected by centrifugation and re-seeded in fresh medium at 2×10^5 cells/ml. Cell viability, as assessed by trypan blue exclusion, was equal to or higher than 99% in all the experiments.

2.3. Immobilization of human cells

U937 cells were immobilized in agarose (Fluka), which is more permeable to oxygen than agar. The consequent higher oxygen diffusion facilitates metabolic activities and therefore cellular respiration and viability. At the time of each experiment the cells were centrifuged at 120*g* for 10 min and resuspended in fresh medium at the desired concentration.

To prepare the agarose plate, U937 cells were inoculated in RPMI 1640 culture medium enriched with agarose. Glucose was also added to preserve the proper osmotic pressure. The culture medium (3.75 ml), containing 100×10^6 cells, was added with 1.25 ml of a previously melted (kept at 42 °C) solution of agarose (4%) and glucose (0.30 M). The mixture was thoroughly shaken, immediately poured in a Petri dish, and let to cool down at 37 °C. A final 1% agarose concentration and a density of 20×10^6 cells/ml were thus obtained. The agarose plate obtained following this procedure had to be daily prepared and stored at 37 °C. It is notable that the cellular density used allows the preparation of a Petri dish suitable for up to five experiments; use of cells in liquid medium requires a much higher number of cells: in fact, preliminary experiments, indicated 200×10^6 suspension cells as necessary for each measurement.

2.4. Biosensor and signal transduction

The agarose plates were appropriately carved to achieve agarose disks ($\varnothing = 1$ cm, thickness = ~0.1 cm) to couple to the sensor. Carved disks from the same agarose plate were separately treated to obtain exposed and unexposed cells. To attain an exposed sample, a disk is merged in a solution, with the proper osmotic pressure and pH, containing the dose of the chemical. The unexposed sample is subjected to the same procedure, but omitting the chemical.

Following this, the measurement of cellular respiration was performed. The disk was constrained to the gas-permeable Teflon membrane at the top of the sensor (oximeter, Clark type, 97-08-00 Orion Research) by a nylon net and an O-ring (Fig. 1). The assembled biosensor was then immersed in a buffered solution (pH 7.4 ± 0.1) of glucose (0.30 M). Cellular respiration is measured by the oximeter, which couples a working Pt cathode and a reference Ag/AgCl anode (−600 mV) through an epossidic insulating resin. The recorded current rises from the O_2 reduction at the cathode ($O_2+2H_2O+4e^- \rightarrow 4OH^-$) and the anode reaction ($Ag+Cl^- \rightarrow AgCl_{(s)}+e^-$). The current intensity is related to the partial pressure of atmospheric O_2 and a key point in the method is represented by the open system approach. In fact, the open system, at constant temperature, allows the maintenance of the dissolved O_2 level in the bulk of the solution for the duration of the O_2 consumption due to the nutrient oxidation (cellular respiration). The experimental kinetic curve ($ppmO_2$ vs. time, Fig. 2) depends on the cellular respiratory activity during the catabolism of glucose.

The slope of the decreasing tract of the curve ($\delta ppmO_2/\delta t$) depends on the glucose oxidation rate and the number of cells. Oxygen diffuses from the solution to the disk containing the immobilized cells and up to the sensor through the Teflon membrane. When the oxidation rate equalizes the O_2 diffusion rate, a stationary state in the respirometric curve is obtained. As from the kinetic model developed, the oxygen concentration at the stationary phases is stable enough for undertaking measurements. The cathode reaction determines a current intensity linearly correlated to the oxygen concentration. A microprocessor allows to obtain a rapid response and the analyser converts the electric signal in a concentration result (ppm). The kinetic of cellular respiration depends on more than 20 enzymes involved in the glucose aerobic metabolic process (during glycolysis, in the cytosol, and the Krebs's cycle and the oxidative phosphorylation in mitochondria). The registered decreasing in the profile of O_2 consumption (Fig. 2) originates from necrosis, apoptosis, and/or cellular injury also caused by the affection of one or more enzymes (e.g. in mitochondria).

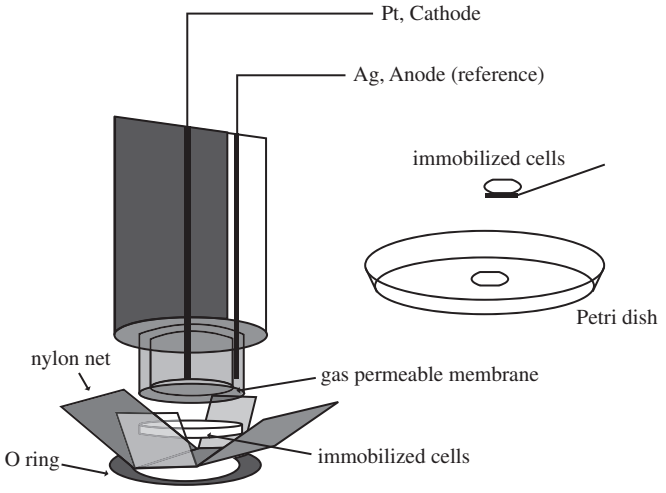


Fig. 1. Scheme of the biosensor assembling.

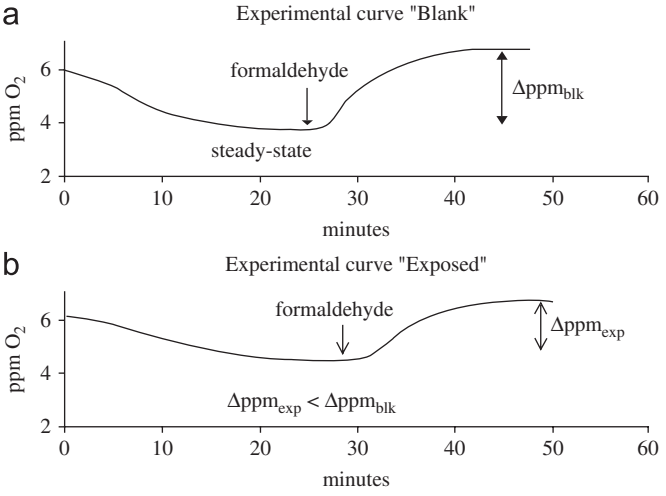


Fig. 2. Example of experimental respirometric curves from immobilized U-937 human cells: (a) unexposed cells (blank), (b) cells after exposure (1 h) to 0.1 mM mercury nitrate.

Calibration of the sensor is performed daily in open air (O_2, about 21%). The biosensor is employed under monitored operative conditions, i.e. solution stirring, temperature, and partial atmospheric O_2 pressure.

2.5. Experimental design and exposure conditions

The bioassay focuses on the enzyme-catalyzed kinetics of respiration and the catalyzer(s) drug rising from the exposure to the toxicant. The typical trend acquired is shown in Fig. 2, where experimental curves obtained before and after cells exposure to 0.10 mM mercury nitrate are reported. When observing the respiration curve, a rapid oxygen concentration decrease is firstly registered. When the rate of oxygen consumption matches the diffusion rate through the disk, the respirometric curve reaches a plateau (steady-state). The subsequent steep increase is due to the cells death deliberately obtained by administration of formaldehyde. The O_2 level (ppm) measured by the biosensor at the final plateau is the O_2 amount in the solution in absence of cellular respiration.

The analytical parameter considered is the variation of O_2 concentration (Δppm) between the two plateaux of the experimental curve (Fig. 2). The toxicological index is calculated as follows: $\delta\% = [1-(\Delta ppm_{exp}/\Delta ppm_{blk})]100$,

where Δppm_{exp} and Δppm_{blk} result from exposed and unexposed cells, respectively. Obviously, the maximum of toxicity is obtained when $\Delta ppm_{exp} = 0$ (δ% equal to 100), while the chemical does not have effect on cell respiration (δ% equal to 0) when $\Delta ppm_{exp} = \Delta ppm_{blk}$. The percentage of respiration inhibition was determined and δ_{50}% values were extrapolated from the dose-effect curve (Fig. 3) for each chemical.

Individual carved disks with entrapped cells were exposed to different concentration of each chemical for the duration of 1 h. The pH value was measured and buffered at 7.4 (HEPES), so as to preserve cellular viability. In the case of clofibric acid, a 15% EtOH solution was used to dissolve the chemical. Also in this case, the blank was prepared and assayed following the same procedure, but omitting the chemicals. After exposure, each disk is coupled to the sensor for the measurement.

Measurements were performed in an open system under thermostatic (37.0 ± 0.1 °C) conditions. The biosensor was immersed in 15 ml solution containing 0.30 M glucose and HEPES buffer 0.01 M (pH 7.4 ± 0.1). Once reached the first kinetic stationary state (Fig. 2), 0.30 ml of formaldehyde was added to stop respiration by killing all the cells. The experimental design covered magnitude (reported doses), duration (1 h), and frequency (once) of exposure.

Human cells required more stringent operative conditions (sterility, osmotic pressure, pH) if compared to yeast cells. In particular, due to the absence of the cellular wall, a glucose solution 0.30 M was used to ensure isotonic conditions between cytoplasm and external solution as well as to minimize the effect of concentration variations on the kinetic of glucose combustion (aerobic respiration); in the experiment with yeast cells, a glucose solution up to 1 M may be used instead.

3. Results

Respirometric bioassays performed on human cells incubated with benzalkonium chloride, clofibric acid, diclofenac, mercury ion, ofloxacin, and sodium dodecyl sulphate showed a significant dose-dependent inhibition of respiration with a marked decline in the rate of respiration occurring after 1 h of incubation. The dose–response data and the relative curves obtained for each chemical are shown in Table 2 and Fig. 3.

For each chemical, four different doses were assayed on human cells so as to draw the toxicity curves (dose is plotted on the *X*-axis, δ% on the *Y*-axis). From dose–response curves, equations with squared correlation coefficients (R^2) ranging 0.91–0.99 were calculated (Table 3).

The slope of the dose–response curve shows how fast the response increases as the dose increases, i.e. the more potent the substance the steeper the curve is. The slope also provides information on the width of the dynamic range of responses that can be achieved. The sharpest increase in the slope of the curve was observed for benzalkonium chloride, followed by mercury nitrate and sodium dodecyl sulphate. A relatively flat slope is obtained for diclofenac, clofibric acid, and ofloxacin, suggesting that the effect of dose increase is minimal for these substances.

The concentration commonly used as a measure of drug potency, i.e. the value at which 50% of the maximal effect is observed (EC_{50}), was derived from each curve along with the corresponding δ_{50}% value. Table 2 reports the mean δ% values, calculated from five replicates (each run on a different day), at four different doses. A relative standard deviation (RSD) less than 8% was found for each dose.

The δ_{50}%-based score we obtained for sodium dodecyl sulphate, mercury nitrate, and benzalkonium chloride was in good agreement with results of a previous investigation on yeast cells (Campanella et al., 1995) and with the available LD_{50}-based score (Table 4). For ofloxacin, clofibric acid, and diclofenac the relative ratios between δ_{50}% values and between LD_{50} values were calculated to obtain preliminary quantitative information. As shown in Table 5 and Fig. 4, LD_{50} ratios are approximately half the value of δ_{50}% ratios.

4. Discussion

The present study substantiates respiration as a sensitive marker of effect of toxicants as well as the performances of the biosensor-based bioassay previously applied on yeast cells. In previous studies, we tested effects of several chemicals on *S. cerevisiae* cells as these are eukaryotes highly specialized in biochemical functions and representing a good model for fast toxicity assessment of chemical agents.

Toxicological results for mercury nitrate, benzalkonium chloride, and sodium dodecyl sulphate were consistent with previously obtained data on *S. cerevisiae* yeast cells (Campanella et al., 1995). However, U-937 cells showed a slightly higher susceptibility, probably related to the absence of cellular wall that allows an easier interaction with the plasmatic membrane.

The resulted inhibition of cellular respiration demonstrates that the chemicals, at the dosage we used, are capable of decreasing metabolic activity, via a mechanism likely involving the inhibition of mitochondrial functions and/or of enzyme(s) engaged in cellular respiration; reduced cell viability may also contribute to the net effect.

Consistency of toxicity ranking scales between human and yeast cells was also verified. In both cases, the toxicity rating scales turned out to be as follows: sodium dodecyl sulphate < mercury nitrate < benzalkonium chloride. When comparing the

Table 2
Mean δ% values (five replicates run on different days) for each dose supplied to the U-937 cells

Concentration (mM)	δ% (mean value, RSD% ⩽ 8)					
	Mercury nitrate	Benzalkonium chloride	Sodium dodecyl sulphate	Diclofenac	Clofibric acid	Ofloxacin
1.00				86		72
0.90						65
0.60			65			
0.50				70	62	32
0.40			43			
0.30	74		37		52	21
0.25	60		26	57	47	
0.20	50					
0.10	23				39	
0.070		82				
0.060		70				
0.030		56				
0.015		26				
0.010				49		

A relative standard deviation (RSD) less than 8% was found for each dose.

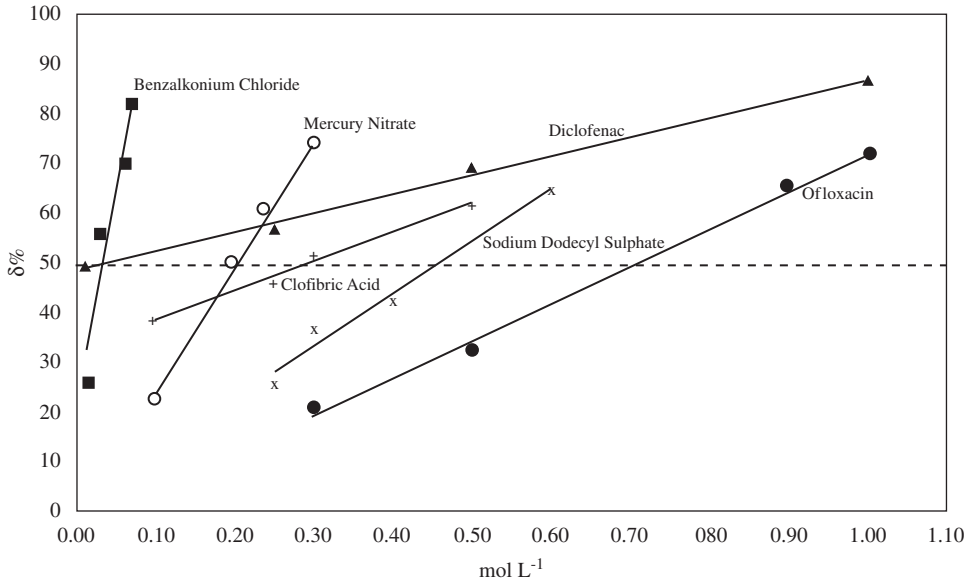


Fig. 3. Dose-effect tendency lines for human cells exposed to benzalkonium chloride, clofibric acid, diclofenac, mercury ion, ofloxacin, sodium, and dodecyl sulphate. Each point is obtained from five independent replicates run on five different days.

Table 3
Figures of merit of the dose– response curves

Chemical	Equation ($y = \delta\%$, x (mmol/l)	R^2	δ_{50}% (mM)	δ_{50}% (ppm)
Ofloxacin	$y = (75.0 \pm 3.6)x - (3.1 \pm 2.7)$	0.995	0.708	255.9
Sodium dodecyl sulphate	$y = (104.9 \pm 11.3)x + (2.1 \pm 4.6)$	0.977	0.457	131.8
Clofibric acid	$y = (58.0 \pm 3.8)x + (33.3 \pm 1.2)$	0.991	0.288	61.8
Mercury nitrate	$y = (252.0 \pm 9.1)x - (1.8 \pm 2.0)$	0.977	0.206	70.6
Benzalkonium chloride	$y = (900.3 \pm 196.0)x + (19.1 \pm 9.6)$	0.914	0.0343	12.0
Diclofenac	$y = (37.7 \pm 4.9)x + (49.0 \pm 3.3)$	0.983	0.0265	8.4_3

Table 4
Comparison of toxicological scales: δ_{50}%, on human cells, δ_{50}%, on yeast cells, and oral LD_{50} on rats (obtained from MSDSs)

Chemical	δ_{50}% (mM) human cells	δ_{50}% (mM) yeast cells	LD_{50} (mg/kg)
Ofloxacin	0.708		3500 (Exocin)
Sodium dodecyl sulphate	0.457	3.66_4	1300
Clofibric acid	0.288		897
Mercury nitrate	0.206	0.426	26
Benzalkonium chloride	0.0343	0.108	240
Diclofenac	0.0265		53

Data confirm the consistency of the chemicals' ranking with the only exception for mercury ion.

Table 5
Tentative quantitative approach

B	A		
	Ofloxacin (3500)	Clofibric Acid (897)	Diclofenac (53)
Ofloxacin (3500)	1		
Clofibric Acid (897)	0.260	1	
Diclofenac (53)	0.0151	0.0596	1
B	A		
	Ofloxacin (0.708)	Clofibric Acid (0.288)	Diclofenac (0.0265)
Ofloxacin (0.708)	1		
Clofibric Acid (0.288)	0.407	1	
Diclofenac (0.0265)	0.0374	0.0920	1

LD_{50} and δ_{50} ratios (B/A) are calculated from data in Tables 1 and 3.

LD_{50}-based toxicity rating scale, an inversion is observed, with mercury ion being more toxic than benzalkonium chloride and diclofenac.

The results suggest that chemicals caused a time-dependent decrease in cellular O_2 utilization rates, which appeared to be responsible for the rise in O_2 tension. The curves are usually qualitative, even though they may refer to *semi*quantitative information. The tentative quantitative approach based on the comparison of toxicity data ratios for δ_{50} and LD_{50} gave preliminary satisfactory results (Table 5 and Fig. 4).

As shown in Fig. 3, it is noticeable that the dose–response curves for diclofenac and clofibric acid do not pass through the zero point. This may suggest some dose-related changes of mechanisms of action; in particular, a switch phenomenon may occur depending on the dose. On the other side, a different explanation is assumable, i.e. different mechanisms of action at doses lower than 0.01 and 0.1 mM for diclofenac and clofibric acid, respectively.

Finally, the assay may also contribute to establish, in a fast and efficient way, whether an amount of chemical in a given matrix could be associated with a toxicological effect. In particular, when considering the exposure to APIs, our results demonstrate that 256, 62, and 8 ppm (mg/kg matrix) were sufficient to reduce human cellular respiration by 50% in the case of ofloxacin, clofibric acid, and diclofenac, respectively. As for mercury nitrate,

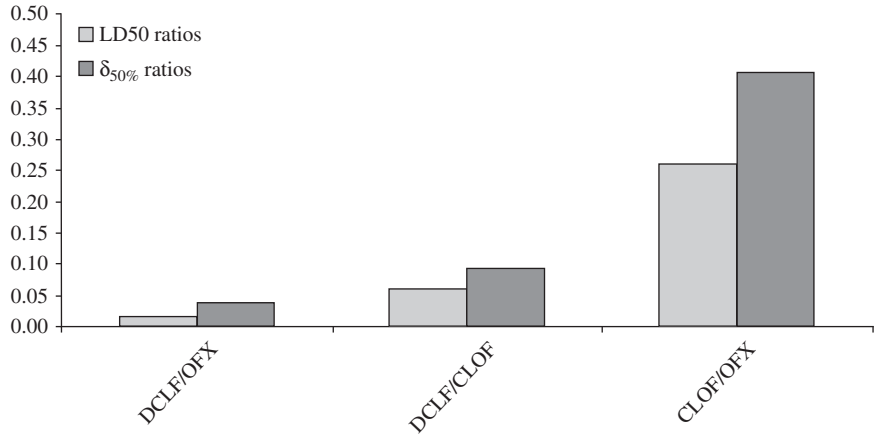


Fig. 4. Tentative quantitative approach. Comparison of LD_{50} and δ_{50} ratios, as detailed in Table 5 (DCLF = diclofenac, OFX = ofloxacin, CLOF = clofibric acid).

71 ppm gave the 50% respiration inhibition on human cells; this finding might deserve further attention in light of the widespread human exposure to mercury ion by means of dental amalgams (Huggins, 2007).

5. Conclusions

The developed bioassay can be useful in understanding chemical-mediated toxicity on human cells; further to yeast cells, the application on human cells confirmed respiration as robust toxicological endpoint to investigate effects on different metabolisms, or rather different reactions to toxicants. The respirometric endpoint is also versatile, in terms of operative modes, so as to fit different needs and scopes. The feed-back to results from application on yeast cells demonstrates good preliminary consistency among toxicity scales on human and yeast cells: if substantiated by further screening applications, chemicals affecting metabolism and mitochondrial functions are likely to give rise to similar toxicity scales on human and yeast cells, and this approach should provide a foundation for extensive studies. In particular, this comparative approach should enable the highlighting of chemicals for which an in-depth study on human cells may reveal particular susceptibility.

The described bioassay confirmed biosensors as cost-effective tools to support toxicological exposure assessment of environmental chemicals; such information is often needed to build health risk assessment scenarios for either environment, consumer products, foods or feeds. The chance of undertaking specific dose–response studies on human cells represent a step towards the minimization of adoption of uncertainty factors to extrapolate findings from *in-vitro* assays as well as towards the development of highly attractive *in-vitro* experimental approaches in view of the increasing demands to refine, reduce or replace animals in laboratory experiments.

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