ECOTOXICITY IN WASTE WATERS AND NATURAL WATERS

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INNOVA-MED

Course on Innovative Processes and Practices for Wastewater treatment and Re-use

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OVERVIEW

- **Terminology**
  Bioassays and Biomarkers
- **Regulatory perspective**
- **Water bioassays: Historic and actual approaches**
  Types of bioassays:
  - Classic approaches
  - Intelligent bioassays
    - Biosensors

Environmental Problems in Water (Trace Organics)

The increasing amount of chemicals in water force to measure:

- Whole biological effects
- Identification and quantification of compounds at trace and ultra trace concentration

- Need of fast methods of analysis “ALARM”
- Need of cheap methods “MONITORING”
- Need of “CHEMICAL ANALYSIS/EFFECTS”

BIOASSAYS

- **The goal of ecotoxicity is to understand how chemicals produce a damage in some organisms, which organisms will be affected, and how this affects the whole receptor environment**
- **Toxicity can be defined as the degree to which a chemical substance elicits a deleterious or adverse effect upon the biological system of an organism exposed to the substance over a designated time period**
- **Aquatic toxicity, genotoxicity and estrogenicity are different expressions of toxicity.**

TOXICITY BIOASSAYS

Bioassays can provide a measure of the whole-effect, produce for a complex mixture integrating different factors, such as: pH, solubility, antagonism or synergism, bioavailability, etc.

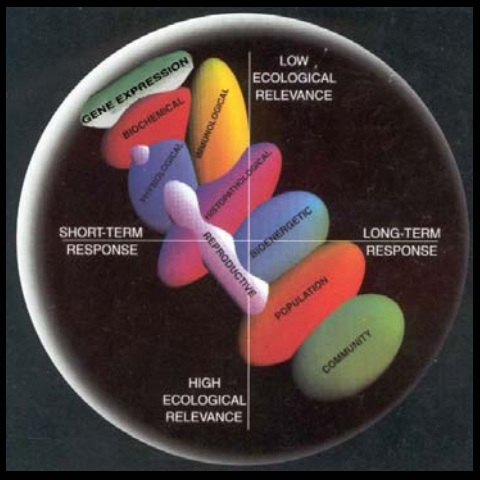
The biological response induced by a substance in different test organisms is diverse.

The use of a battery of bioassays involving different species at different trophic levels is an efficient and essential tool for predicting environmental hazards to the aquatic ecosystem.

TERMINOLOGY

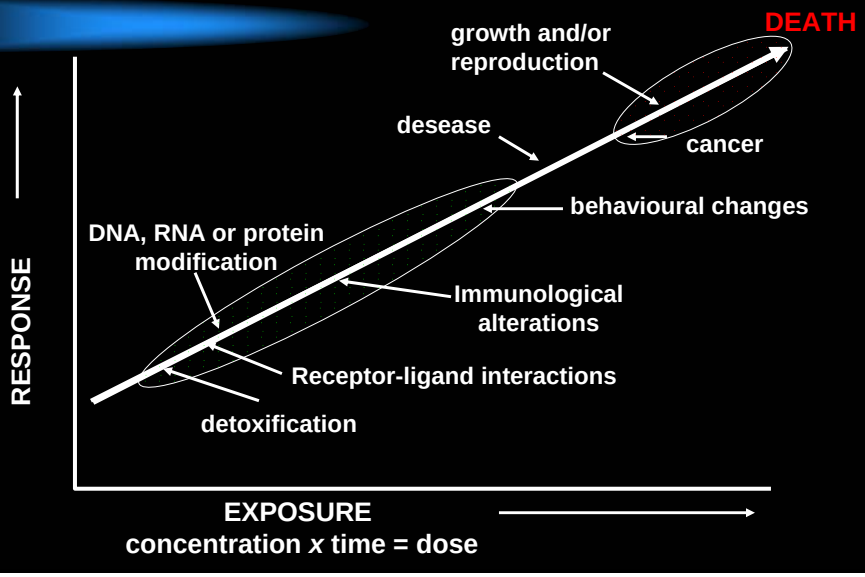
BIOMARKER	≠	BIOASSAY
Measurement of possible toxic responses in test organisms collected from the environment		Measurement of toxic responses after exposure under controlled conditions in the laboratory. In general using cultured organisms

Biomarkers at various organizational levels



1. Molecular
   - DNA adduct or integrity
   - Binding to receptor
   - Alteration of cell structural elements
2. Biochemical
   - Changes in gene expression
   - Induction of enzymes or stress proteins
3. Physiological, bioenergetic and reproductive
   - e.g. Inhibition of growth, and/ or reproductive output
4. Histopathological
   - e.g. Tissue damage; Imposex
5. Behavioural

BIOASSAYS – TOX END-POINTS



TERMINOLOGY

in vivo bioassays
vs.
in vitro bioassays

short-term or *accute* bioassays
vs.
long-term or chronic bioassays

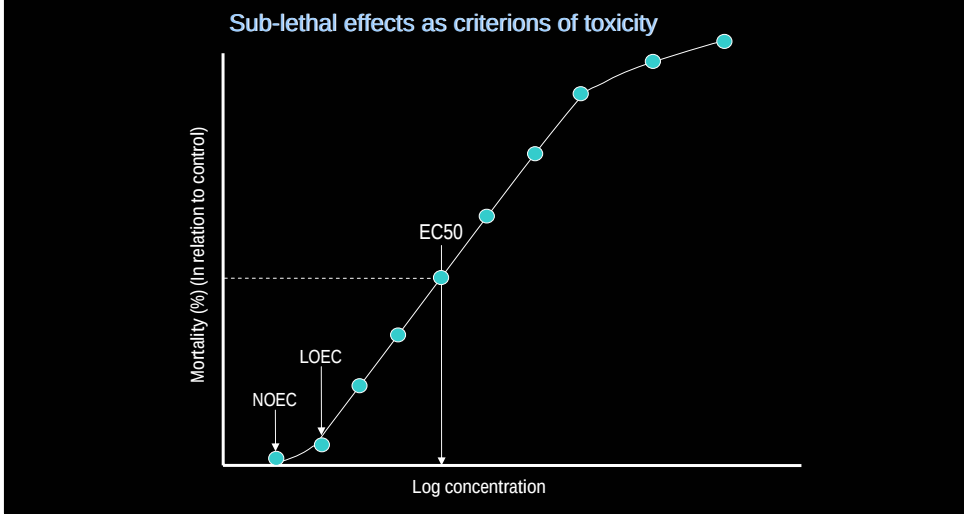
predictive bioassays
vs.
assessment bioassays

single species
vs.
many species
bioassays

"BATTERY"

single vs. multiple
end-points

BIOASSAYS – TOX END-POINTS



Regulatory persective – statutory pollution control

An ideal bioassay should be:

- Reliable and reproducible;
- Economical of time and resources;
- Able to yield statistically robust data;
- Relevant, practicable and readily understood by the layman;
- Able to utilize test organisms from a reliable stock;
- Simple to emulate;
- Regularly intercalibrated;
- With a clearly defined end-point;
- Sensitive to a wide range of pollutants.

EFFLUENT BIOASSAYS

Overview – the last 30 years...

10 years ago

- **> 90% studies used predictive, single-species bioassays;**
- **> 75% of acute tests; Mortality tests**
- **The most frequently used organisms:**
  - **- invertebrates (75%)**
  - **- fish (23%)**
- **Chronic and sub-lethal effects – less than 2% studies.**

Today….Shift from the whole organism biotest to "micro-scale" tests and in vitro bioassays

- Rapid;
- Less expensive;
- Suitable for screening;
- Can be efficiently used to direct chemical analyses in the Toxicity Identification Evaluation (TIE) procedures (so-called Effects Directed Analysis – EDA);
- Minimize the "animal testing" (Europe).

UK DTA – *"The simplest predictable form of life should be used for ecotoxicity testing in direct toxicity assessment, i.e. bacteria, plants or invertebrates should be used instead of vertebrates."*

Germany lab guidelines – DIN standardized fish microplate embryo toxicity test should be used instead of the whole organism fish toxicity test.

"In vivo" vs "In vitro"

- *In vivo* studies are very important both in the field and laboratory (for validation), they are based on a wide variety of end points, including cell differentiation and enzyme activities. However, it is not possible to use *in vivo* methods for routine or monitoring studies: ethical problems, expensive, time consuming, and big installations (aquariums,..) are neded.

- *In vitro* bioassays can be performed more quickly, these tests are much more cost-effectives than *in vivo* assays. However, *in vitro* assays are not able to explain all the mechanisms.

Aquatic toxicity bioassays can be classified according to the test species involved.

- Fish
- Invertebrate

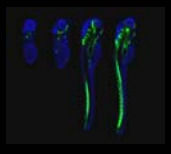
→ Classic "In vivo" toxicology tests

- Plant and algae bioassays
- Whole-cell, Bacteria
- Cellular organelles
- Biochemical reactions
- DNA, RNA

"In vivo" Fish toxicity bioassays

End Points:

- Mortality: Routinely used, fish-lethality assays involve exposure to the toxicant for a maximum of 96 h. The results are reported as the percent volume that is lethal to 50% of the organisms within the prescribed period of time (LC_{50}).
  - static and
  - flow-through
- Larval growth
- Larval survival
- Reproduction



Rainbow Trout
(Oncorhyncus mykiss)

Fathead minnow
(Pimephales promelas)

Zebra fish
(*Danio reiro*)

"In vivo" Fish toxicity bioassays: Estrogenicity

In vivo assays for estrogenicity are widely used.
They are based on a wide variety of end points, including cell differentiation and enzyme activities.

Example: In vivo medaka screening bioassay
(Nimrod and Benson, 1998 ; Thompson *et al*., 2000).

Randomly selection of adult Japanese medaka, *Oryzias latipes* from a stock culture

Exposure the animals to different concentrations in controlled conditions (Ex. 24 h static water renewal, etc..), during established times of exposure (7 days..)

Collection of livers and plasma from fish

Vitellogenin (VTG) analysis by means of Immunoassay or any other analytical approach.

"In vitro" Recombinant yeast assay

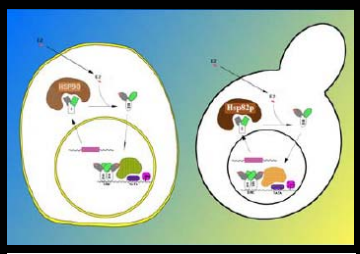
This assay is based on the evaluation of the potential of a compound to interact with estrogen receptor and activate hormone-regulated gene-promoters.

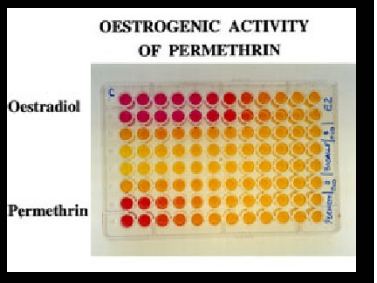
Yeast reporter assay is based on a two-hybrid system.

Beta-galactosidase, has been used as the most common reporter enzyme.

96-well microtiter plates

3h-3days

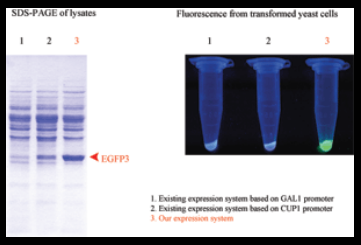




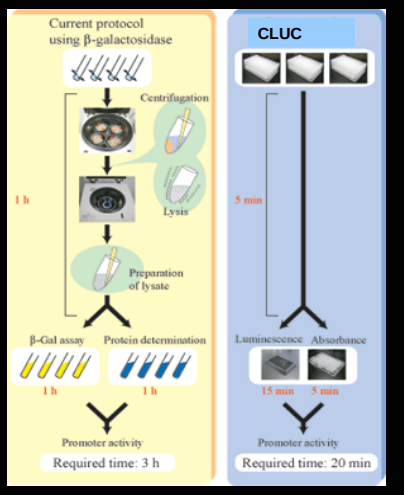
Garcia-Reyero, Natàlia et al. (2001) Environmental Toxicology and Chemistry, Vol. 20, No. 6, pp. 1152–1158, 2001

"In vitro" Recombinant yeast assay

Novel yeast reporter assay are more suitable for high-throughput analysis, employing in the reporter assay luciferase, named CLuc, as a reporter enzyme.



Nagao, A., Ohmiya, Y., and Ohgiya, S. (2007) Yeast mutant with efficient secretion identified by a novel secretory reporter, Cluc. *Biochem. Biophys. Res. Commun., in press.*



Invertebrate bioassays: Daphnids

Chronic toxicity test using macro invertebrates have been extensively used in aquatic risks assessment studies.

The parameters measured are mortality or reproduction

One of the most common invertebrate toxicity tests uses *Daphnia* and *Ceriodaphnia*, both freshwater species pertaining to *Cladocera*. Tests are carried out by exposing the test organisms to toxic substances under control conditions.

Acute lethality tests with Daphnia conducted for 21 days are well established and standardized

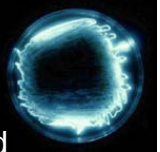
Daphnia magna

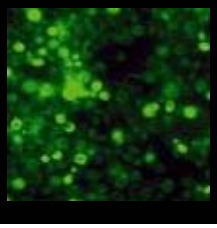
Cerodaphnia

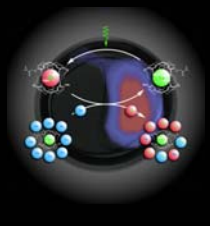
INTELIGENT BIOASSAYS AND BIOSENSORS

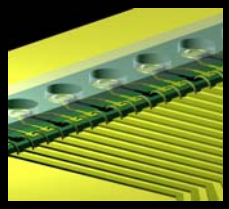
Main advantages:

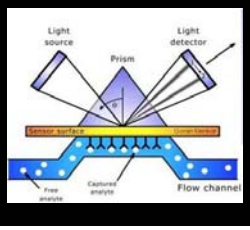
- Rapid responses
- Cost effective
- No higher animal are involved
- Easy interpretation
- Miniaturization
- Automatization
- On-line
- Remote controlled











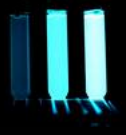
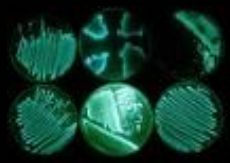
Plant and algae bioassay

Diverse toxicity test based on algae have been developed.

- Test species, such as marine unicellular algae *Selenastrum capricornutum* or *Dunaliella tertiolecta* are used as indicator species.
- Inhibition of algal growth is used as the indicator of toxicity.
- The main disadvantages of algal methods is a lack of reproducibility between consecutive assays.

Bacterial toxicity assays

The more widely used bioassays in routine laboratories for evaluating water toxicity are based on inhibition of the bioluminescence of marine bacteria.
The better-known species of luminescent marine bacteria are ***Vibrio fischeri*** and ***Photobacterium phosphoreum***, which naturally emit light due to an enzyme, the bacterial luciferase. Any substance that affects the bacterial metabolism produces a proportional inhibition of the luminescence.

Bacterial toxicity assays: Bioluminescence inhibition

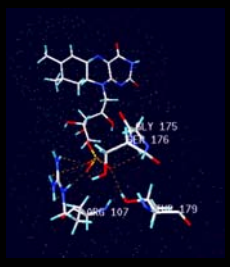
The use of luminescence organisms to assess toxicity has been known for more than 40 years (Serat et al., 1965) [ii]. In 1979 a toxicity bioassay using luminescent bacteria was developed by Bulich et. al. [iii] to assess toxicity of wastewater effluents and industrial discharges. This technique allows the easy screening of large numbers of aqueous samples in a quick, reliable, and inexpensive way. This toxicity assay was commercialized for first time by Microtox and described in Beckman's Operating Manual [iii].
During the last decade, interest has increased in the ecological characterization of real samples by means of combined protocols, involving **chemical analysis** and **toxicological evaluation**. These methods combine the advantages of the diagnostic methods, for which previous information about the sample is not necessary and report of an ecological global effect, and those of targeted quantitative analysis.

[ii] W.F. Serat, F. E. Budinger, P. K. Mueller. J. Bacterial. **90** (1965)832-833.
[iii] A.A. Bulich, 1979. Use of luminescent bacteria for determining toxicity in aquatic environments, P. 98-106. In L. L. Markings and R. A. Kimerle eds, Aquatic Toxicology, ASTM 667. American Society for Testing and Materials, Philadelphia, PA.
[iii] Beckman Instruments, Microtox system operating manual, Beckman Instruments, Inc., Carlsbad, CA, USA, 1982.

Bacterial toxicity assays: Bioluminescence inhibition

Vibrio fischeri is a common marine organism and can routinely be isolated from fresh fish.

Photobacterium phosphoreum *(Vibrio phosphoreum)* is another type of marine bacteria. 1-7 day old colonies grown at 20 degrees Celcius, exhibit extremely bright luminescence.

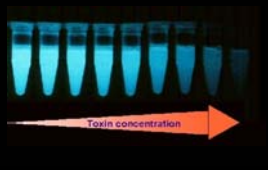


Bacterial toxicity assays: Bioluminescence inhibition

These marine bacteria naturally emits light, thanks to an enzyme the bacterial ***luciferase***, which catalyses the following reaction:

$FMNH_2 + O_2 + R\text{-}CO\text{-}H \longrightarrow FMN + R\text{-}COOH + H2O +$ **LIGHT**

The light production is directly proportional to the metabolic status of the cell, and any inhibition of cellular activity is reflected in a decrease in bioluminescence.
The inhibition percentage (%I) is determined by comparing the response given by a saline control solution to that corresponding to a sample.



Bacterial toxicity assays: Bioluminescence inhibition

- Toxicity is expressed as EC_{50}, which is the effective concentration of a toxic substance producing the 50% of light reduction.
- Luminescence tests have the advantage of being rapid, sensitive and reproducible.
- This is a standard method for aquatic toxicity

- *V. fischeri* is a marine bacteria so, for the good performance of the should be carried out using a 2% of saline solution.
- Because of the salinity, the insolubility of some organic substances is enhanced, thus producing turbid solutions.

Bacterial toxicity assays: Bioluminescence inhibition

Microtox® assay

Abratox Camera

Vibrio fischery

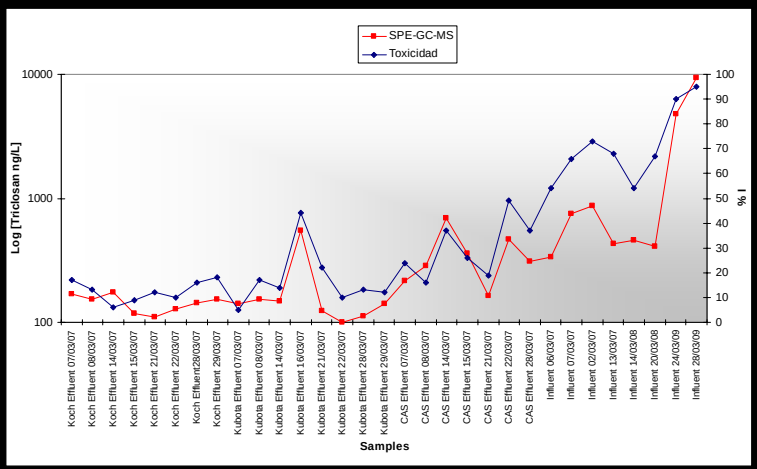
Size: 15x10 cm

Unplug

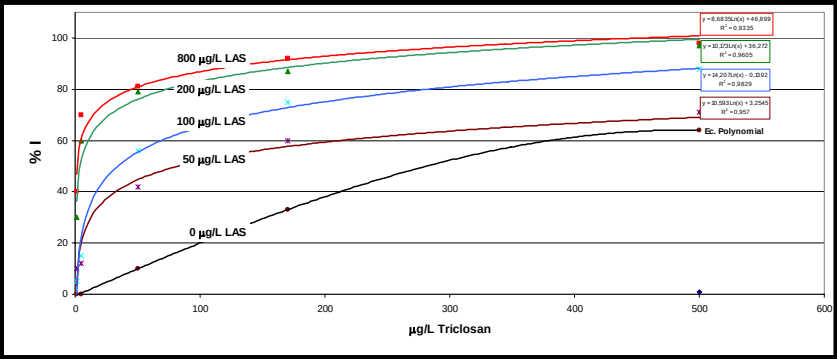
On site measurements

15 min 20 measurements

Bacterial toxicity assays: Bioluminescence inhibition



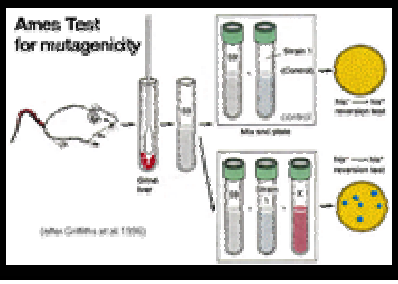
Bacterial toxicity assays: Bioluminescence inhibition



Bacterial toxicity assays: Genotoxicity Ames Test

- Genotoxicity is associated with different structures, such as phenols, chlorophenols, polychlorinated biphenyls (PCBs), or polyaromatic hydrocarbons (PAHs), and constitutes an early screening for possible cancer inducing activity of pollution. Among those based on microorganisms, we would like to emphasize the assays based on the bacteria ***Salmonella typhimurium***.

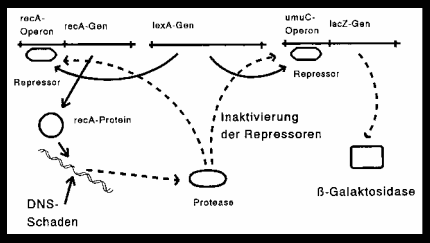
- The most widespread is the **Ames test** [i] that was established as a routine method of analysis. It is based on the retromutation of *S. typhimurium* TA98 (histidine dependent).



[i] B.N. Ames, F.D. Lee, W.E. Durston, Proc. Natl. Acad. Sci. USA 70 (1973) 782.

Bacterial toxicity assays: Genotoxicity
UMU Test

The umu test is also based on genetically engineered bacteria *Salmonella thyphimurium* TA 1535 pSK1002 (gram negative, facultative anaerobic enterobacteriaceae). and the genotoxicity is detected measuring the activation of the bacterial SOS repair response of genetic damage in the bacterium, through measuring b-galactosidase activity [II]. The molecular background and the specific activation cascade of the SOS response genes necessary for the umuC-activation is shown



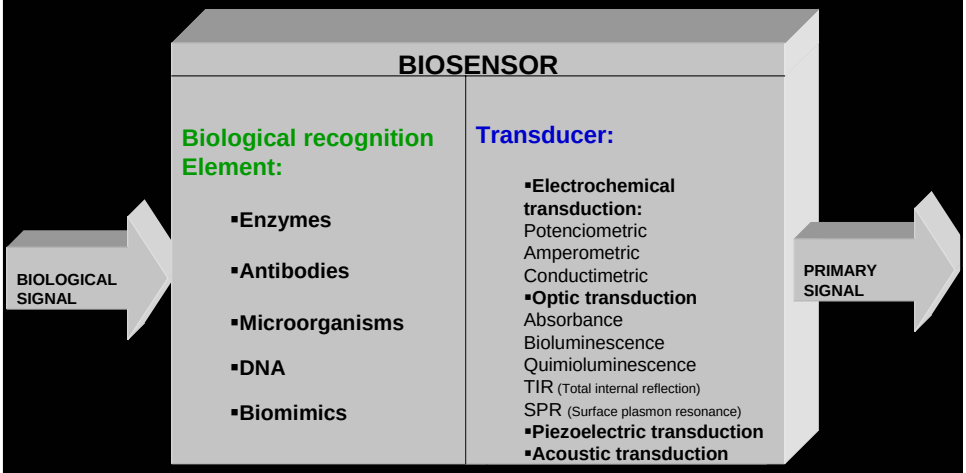
This is a standardized method that is validated [III] for water control

[II] C.T. Kenyon, Trends Biochem. Sci. 8 (1983) 8
[III] International Organization for Standardization, ISO/DIS 13829, Water quality – determination of genotoxicity of water and wastewater using the umu-test, ISO, Geneva, Switzerland, 2000.

Biosensors

A biosensor is defined by IUPAC as a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with a transduction element.

Biosensors



Why Biosensors for Environmental Monitoring ?

- Fast
- Keep it Good but Cheap
- Portable, on-line, at-site, remote configuration
- Early Warning
- Quantitative-Semiquantitative
- Toxic effects
- Complementary to GC/LC/MS

BIOSENSOR-LIMITATION

LACK OF VALIDATION / VERIFICATION

⇓

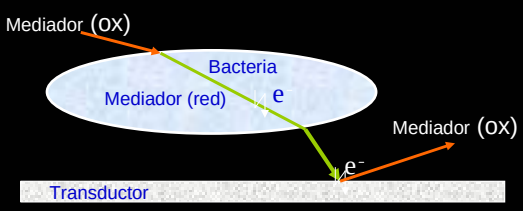
Scientific understanding/technological development are advancing, but **commercialisation is slow**

⇓

VALIDATION IS REQUIRED

Toxicity biosensors

Amperometric sensor
Screen printed electrodes
Bacteria immobilized on the electrodes



The electrode is composed by a reference electrode Ag/AgCl and a graphite working electrode where bacteria are immobilized.

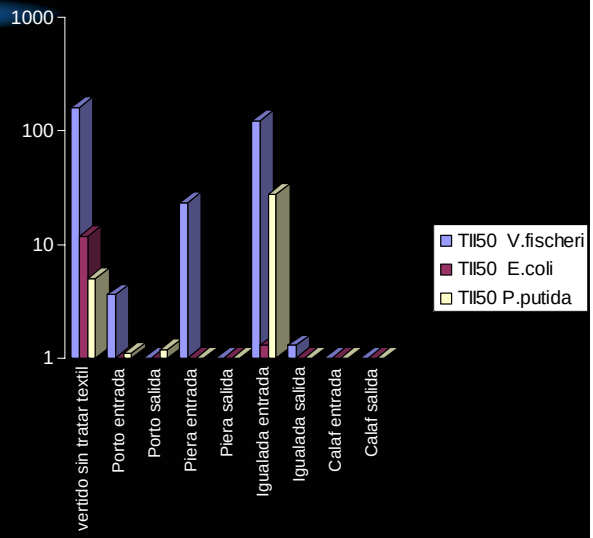
Mediator:
hexacianato ferrico potásico

Example of responses of different organisms vs. Toxic organic compounds in real samples

Escherechia coli (NCIMB 8277)

Pseudomonas putida.

EC_{50}, TU, TII were stablished

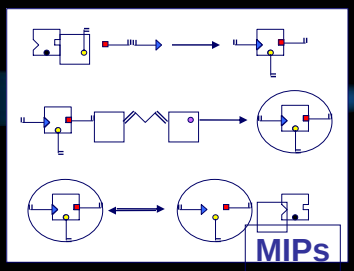


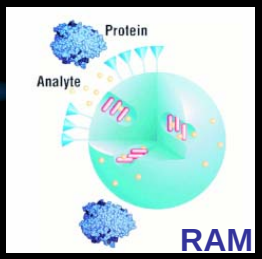
Farré, M. Pasini, O, MC Alonso, M. Castillo, D. Barceló *Anal. Chim. Acta* 426 (2001) 155-165

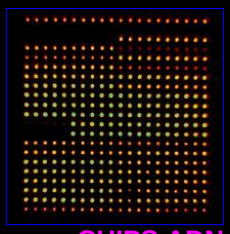
And the PRESENT and FUTURE.....

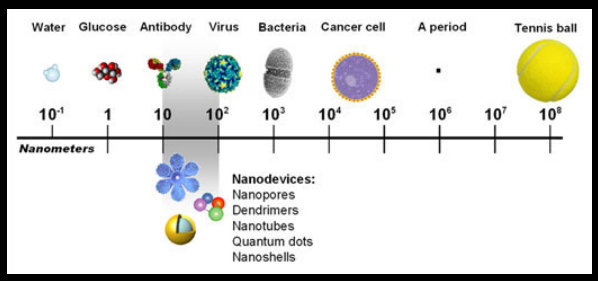
- New materials
- Nanotechnology
- Integration of different technologies

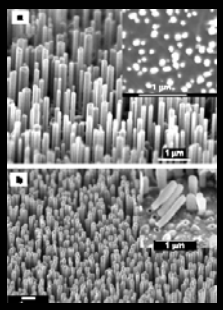
New materials





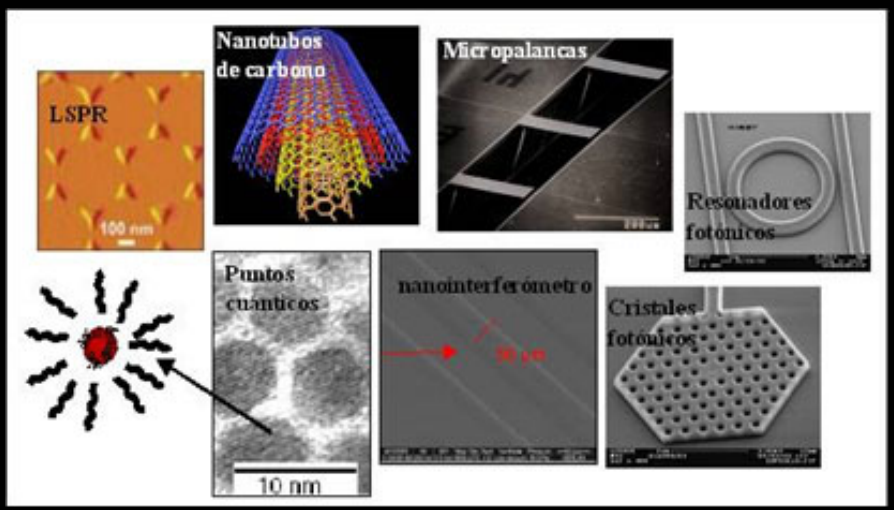






"Single crystal gallium nitride nanotubes", J. Goldberger, R. He, S. Lee, Y. Zhang, H. Yan, H. Choi, P. Yang, Nature, **2003**, 422, 599

NANOBIOSENSORS



RECENT ACHIEVEMENTS

1 New Optical Devices for target pollutants and biological effects: SPR

2 Mass measurements for environmental applications: Quart crystal microbalance

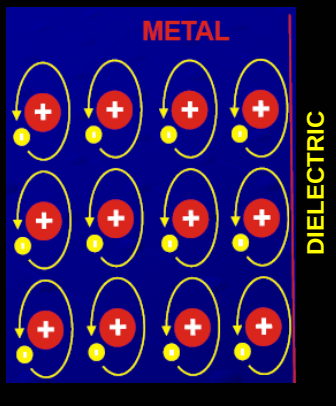
3 Sensors arrays

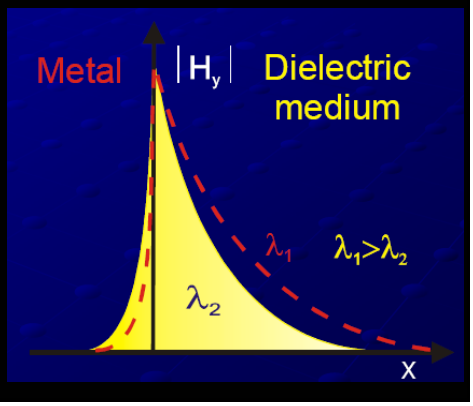
4 Miniaturized Electrochemical devices for biological effects: DNA, Enzymes

Surface Plasmon Resonance (SPR)

Theory

An incident monochromatic light at the interface between 2 substances with different refractive index can produce an evanescent wave



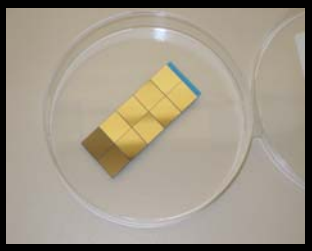


Surface Plasmon Resonance (SPR)

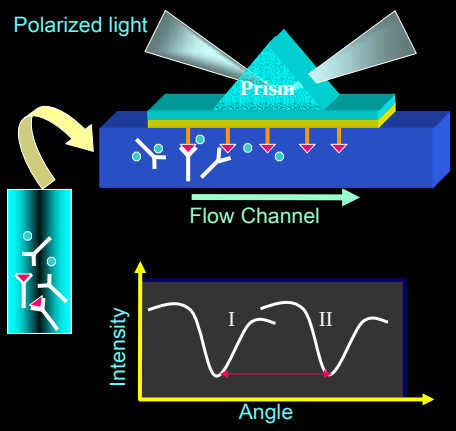
Biomolecular Recognition Elements in Evanescent Field Sensors:

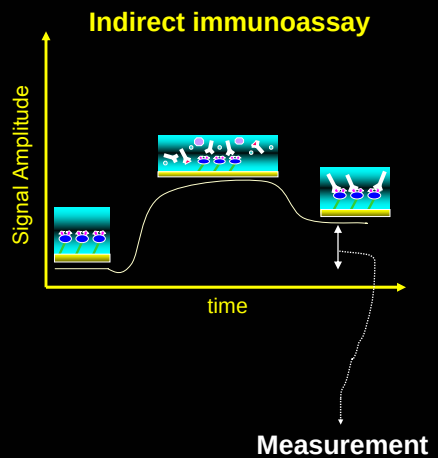
SPR is a generic optical technology that can be combined with specific biological receptors against particular target analytes

- **Antibodies** → **Immunosensor Chips**
- **Proteins**
- **DNA**
- **RNA**
- **MIPs (plastibodies)**



Surface Plasmon Resonance (SPR)

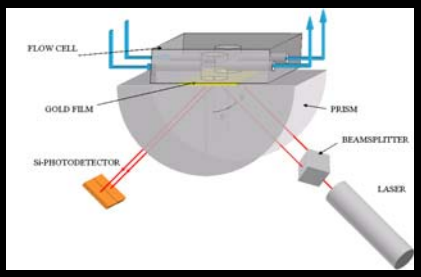




SPR: Different configurations and equipments

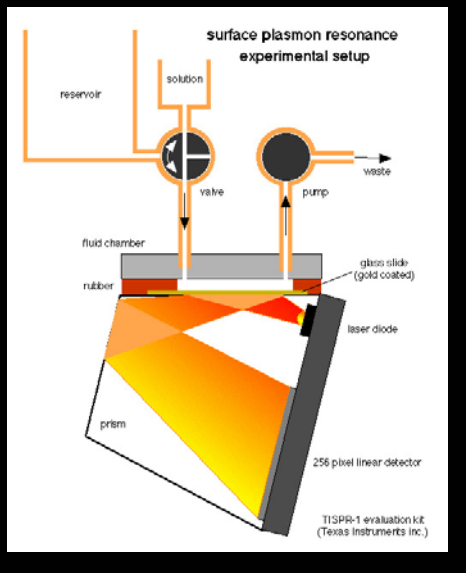
1- Kretschmann: Total internal Reflection

Different commercial optical platforms Biacore, SENSIA…



SPR: Different configurations and equipments

2- SPREETA



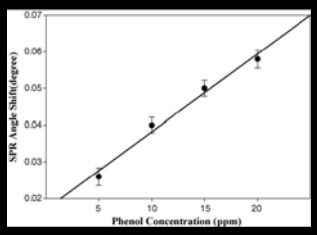
SPR: Examples

Sample	SPR µg/L	SPE-HRGC/MS µg/L	Sum of Triazine µg/L
1	0.05	0.05	0.25
2	0.10	0.08	0.13
3	1.00	0.82	1.07
4	0.26	0.24	0.46
5	0.22	0.19	0.51
6	0.20	0.20	0.59
7	0.11	0.07	0.27

***IC50* = 0.17**
LOD = 0.02 ppb = 20 ng/L
15' without sample enrichment

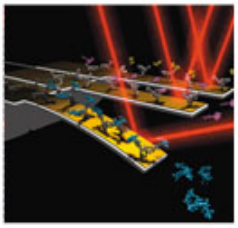
M. Farré, E. Martínez, J. Ramón, A. Navarro, J. Radjenovic, E. Mauriz, L. Lechuga, Mª. P Marco, D. Barceló
Analytical and Bioanalytical Chemistry (2007)

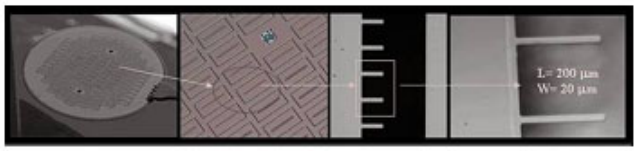
The immobilized cell using self-assembled synthetic oligopeptidewas applied to the biological toxicity detection of environmental pollutant.
Thin films based on cysteine-terminated synthetic oligopeptides were fabricated for the immobilization of *Escherichia coli* O157:H7 on gold (Au) substrate.

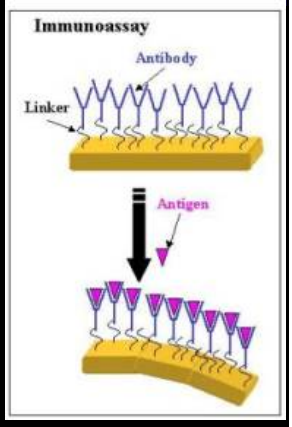


Jeong-Woo Choi*, Kwang-Won Park, Doo-Bong Lee, Woochang Lee, Won Hong Lee
Biosensors and Bioelectronics (2005)

Quartz crystal microbalance







Bisfenol A
Transducción: **Quartz crystal microbalance**
IMMOBILIZATION (2-methacryloyloxyethyl phosphorylcholine (**MPC**) polymer
LOD 0.01 ng/ml WITHOUT SAMPLE PRE-TREATMENT IN WASTEWATER

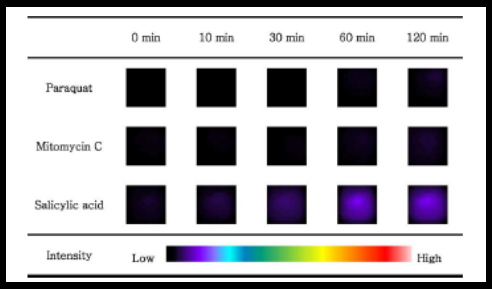
S. Kurosawa, J-W Park, H.Aizawa, S-I. Wakida, H. Tao, K. Ishihara,

Biosensors and Bioelectronics 22 (2006) 473–481

SENSOR ARRAYS

Toxicity measurements using more than 20 different bacterial species genetically modified using the *operon lux*

Bacteria immobilized on sensor chip



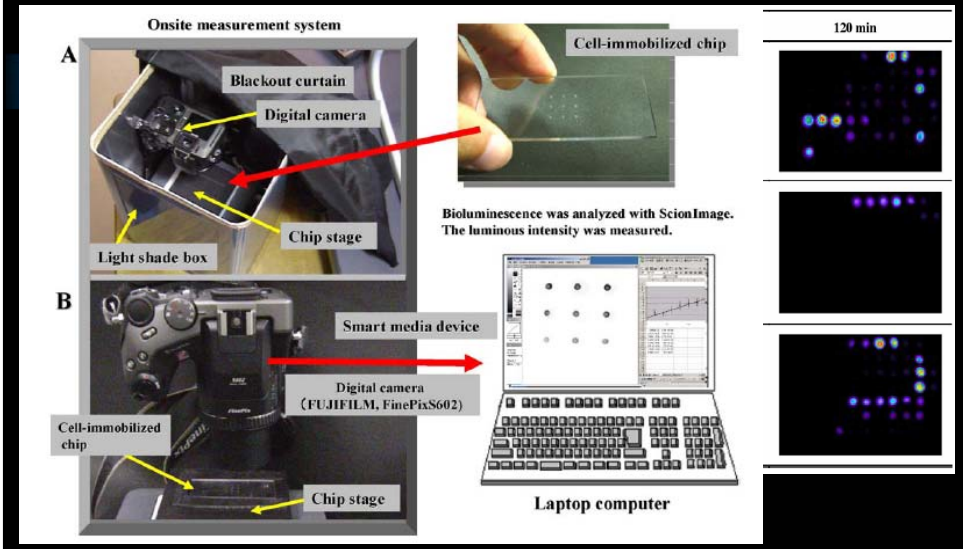
Strains used in this study

Strain	Plasmid	Host	Strain	Plasmid	Host
DS1	*pSodALux(Xl*[a]*)*	RFM443[b]	DK1	*pKatGLux(Xl)*	RFM443
DP1	*pPgi-5Lux(Xl)*	RFM443	NagK	*pNagRLux(Vf)*	P.putidaKCTC1768
EBSoxR	*pSoxRLux(Xl)*	RFM443	TV1061	*pGrpELux(Vf)*	RFM443
EBFumC	*pFumCLux(Vf)*	RFM443	DC1	*pClpBLux(Xl)*	RFM443
EBSoxS	*pSoxSLux(Vf)*	RFM443	DO2	*pOmpTLux(Xl)*	RFM443
EBInaA	*pInaALux(Vf)*	RFM443	BM401	*pLuxRLux(Xl)*	RFM443
EBHmp	*pHmpLux(Vf)*	RFM443	GC2	*pLacLux(Xl)*	RFM443
DPD1710	*pRecALux(Vf)*	JC7623[c]	DRP1	*pRpoSLux(Xl)*	W3110[d]
EBJM2	*pGltALux(Xl)*	RFM443	Kan3	*pKanLux(Vf)*	W3110
DPD2540	*pFabALux(Vf)*	RFM443	RFM443	*N/A*	RFM443

[a] *Vf: lux* genes are from *Vibrio fischeri*. *Xl: lux* genes are from *Xenorhabdus luminescens*.
[b] The genotype of RFM443 is (*rpsL* – (StrR), *galK2*, *lacΔ74*).
[c] The genotype of JC7623 is (AB1157, *recC22*, *recB21*, *sbcB15*, *sbcC201*).
[d] The genotype of W3110 is (F⁻ *lam-In (rrnD-rrnE)1 rph-1*).

J. H. Lee, R. J. Mitchell, B. C. Kim, D. C. Cullen, M. B. Gu
Biosensors and Bioelectronics 21 (2005) 500–507

SENSOR ARRAYS



J. H. Lee, R. J. Mitchell, B. C. Kim, D. C. Cullen, M. B. Gu
Biosensors and Bioelectronics 21 (2005) 500–507

"Fish and Chips"
New approaches – ecotoxicogenomics

DNA-array based technologies, proteome analysis, metabolomics...

Examples - gene or protein signatures used to identify the MOA(s) of a single chemical or complex environmental mixtures.

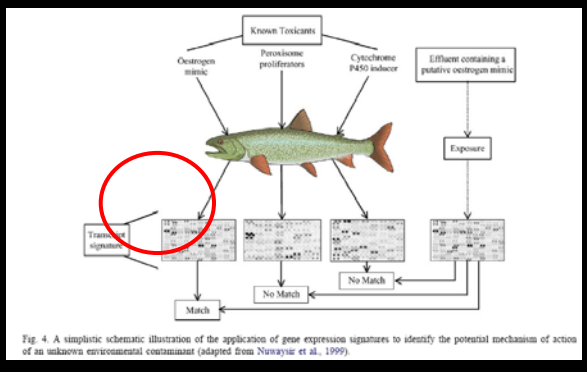
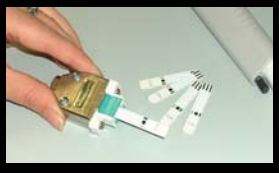


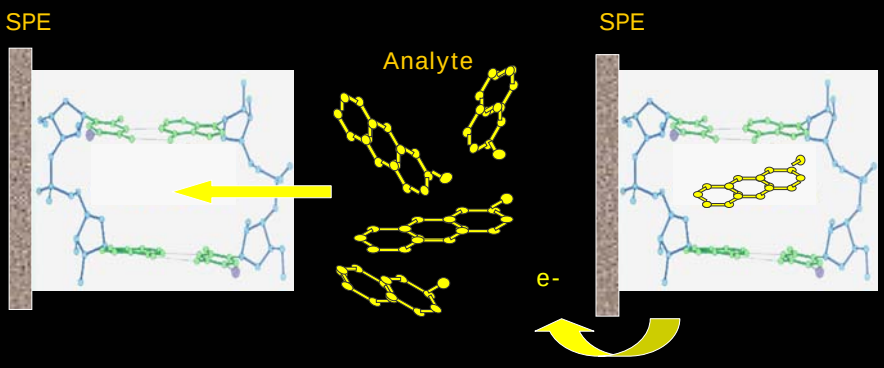
Fig. 4. A simplistic schematic illustration of the application of gene expression signatures to identify the potential mechanism of action of an unknown environmental contaminant (adapted from Nuwaysir et al., 1999).

Miniaturized Electrochemical devices for biological efects:

GENOTOXICITY	**NEUROTOXICITY**
DNA electrochemical biosensor for rapid environmental analysis	**AChE-based electrochemical biosensor for acethylcholinesterase inhibitors detection**



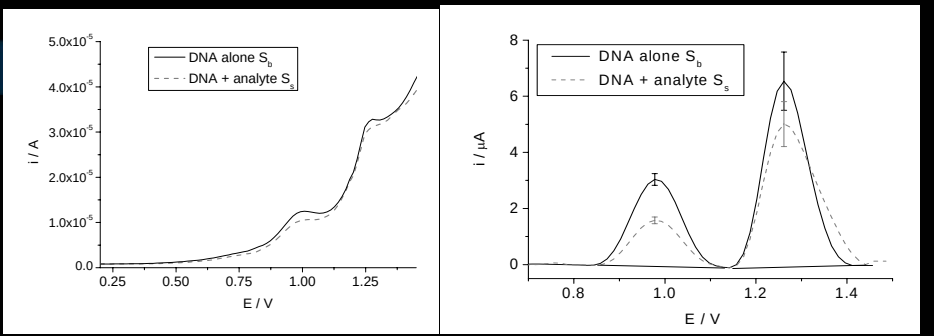
DNA biosensor: principle



I - Molecular recognition system: double stranded DNA

II - The analyte produces a chemical-physical modification of the immobilised DNA. Therefore, this causes a different availability in the guanine oxidation.

DNA biosensor: analytical signal



The interaction of the DNA with pollutant molecules modifies the oxidation of the DNA guanine base

Guanine Oxidation

E_p = +1.0 V *vs* Ag-SPE

Toxicity index:

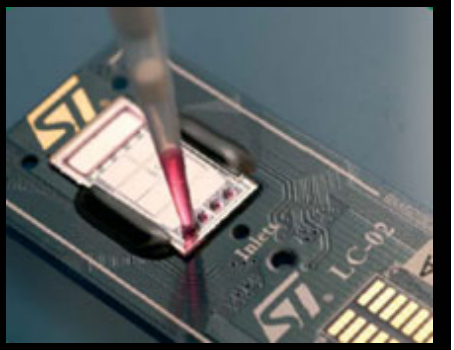
Signal % =(Ss/Sb)*100

Sb: guanine peak after the interaction with the blank solution

Ss: guanine peak after the interaction with the sample

The Future of Biosensors for Environmental Monitoring in EU

- Integration of different technologies
- Complementary measurements
- Reduced size equipments
- Lab on a chip
- Remote control



Acknowledgments

We would like to thanks the European Union through the project **INNOVA-MED** and by the Spanish Ministry of Education and Science through the project **CEMAGUA**.

THANK YOU FOR YOUR ATTENTION