RISK ASSESSMENT OF MIXTURES OF NINE PHARMACEUTICALS USING MULTISPECIES BIOTESTS

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NORMAN Workshop-Mixtures and metabolites of chemicals of emerging concern-18 - 19 November 2009, Amsterdam, The Netherlands

• PHAREM project (www.eng.ucy.ac.cy/PHAREM)

- Development and application of innovative advance oxidation processes for the removal of active organic compounds in urban wastewaters and monitoring of toxicity

- Partners
  - University of Cyprus
  - Salerno University & Naples University, Federico II
  - Aegean University

- **Main tasks**

  T1: Sources and fluxes of active biologically compounds in wastewaters of Cyprus

  T2: Analysis and prediction of active pharmaceutical substances

  T3: Development of methods for assessing WET and mixture toxicity in synthetic samples

  T4: Photocatalysis for removal in synthetic samples

  T5: Ultrasound for removal in synthetic samples

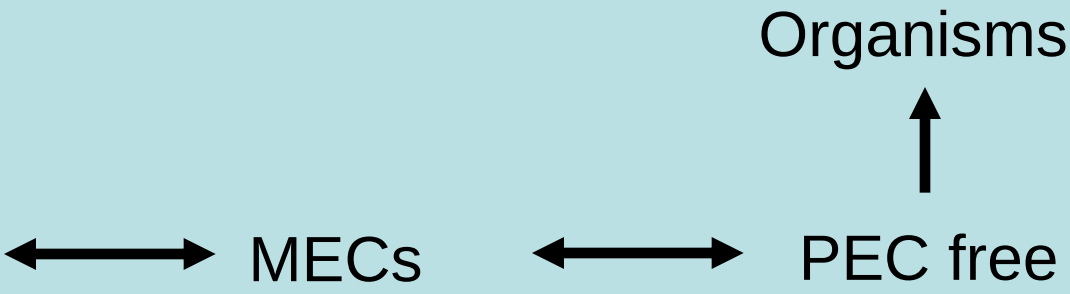
  T6: Combination of methods for removal

T1: Sources and fluxes of active biologically compounds in wastewaters of Cyprus

Germany	USA	Cyprus
Analgesics	Codein and analgesics	Antibiotics
Antidysenterics	SSRI/ SNRI antidepressants	Antidiuretic
Antiinfectives	Cholesterol reducers (statins)	Analgesic/ Antiinflamatories
Antitussiva	ACE inhibitors	B-blockers
Phychiatric drugs	Beta blockers	Antipyretics
ACE Inhibitors/ AT Blockers	Calcium channel blockers	Antihypersentives
Dermatologic Drugs	Oral contraceptives	Antiepileptics
Antihistamines, anti-asthmatic	Proton pump inhibitors	
Antihypersentives	Thyroid, Hormones	
Ophthalmic drugs	Antihistamines	

Risk characterisation of pharmaceuticals in the environment

- PEC influent
- PEC effluent and sludge
- PEC surface water
- PEC sediment
- PEC soil



- **Spatial scale**
- **Watershed of Catchment-Based Environmental Models**
- **PhATE**
- **Boundary condition Model**
- **GREAT-ER**
- **GIS-ROUT**

Concentration prediction and risk assessment for nine pharmaceutical active ingredients in urban wastewater treatment plant effluents, Submitted !!

Despo Fatta-Kassinos , Evroula Hapeshi , Marlen I. Vasquez, Sureyya Meriç

Antiobiotics
Amoxicillin
Sulfamethoxazole
Erythromycin
Ofloxacin

NSAIDs
Diclofenac
Ibuprofen

Beta blockers
Atenolol
Propranolol

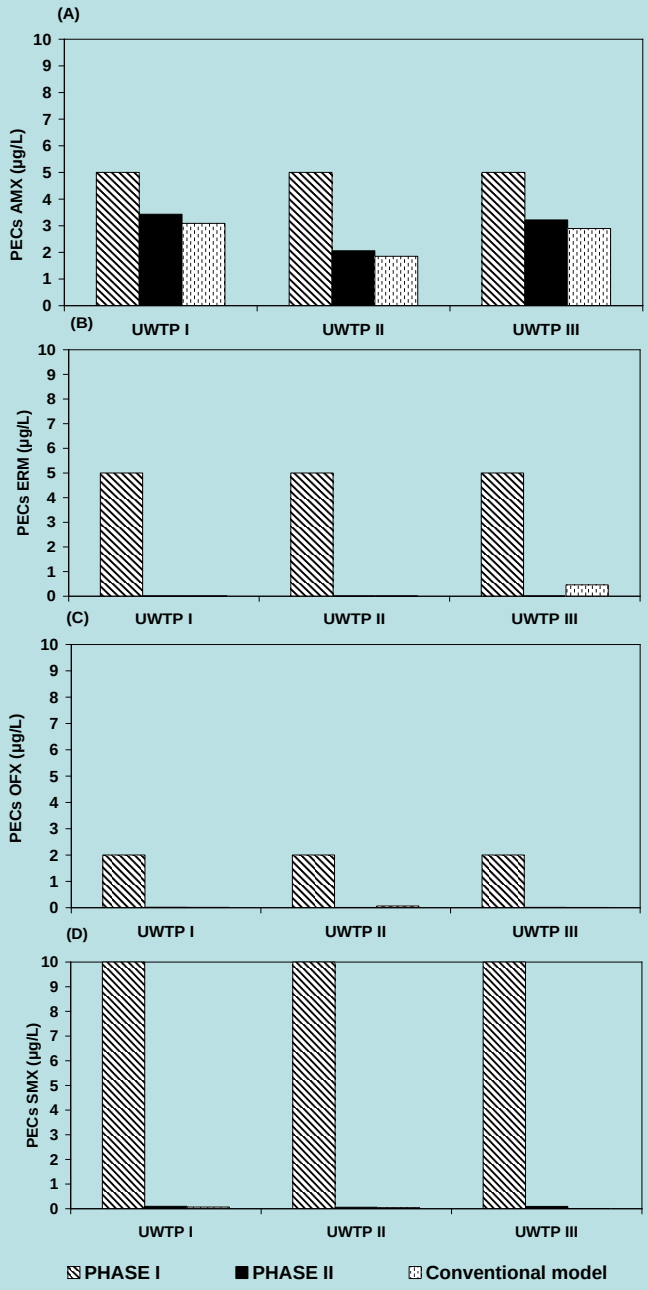
Anti-epilectic
Carbamazepine

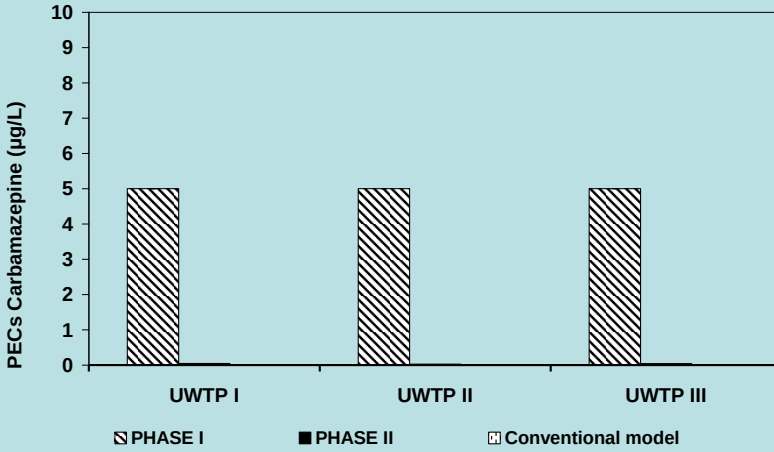
EMEA model

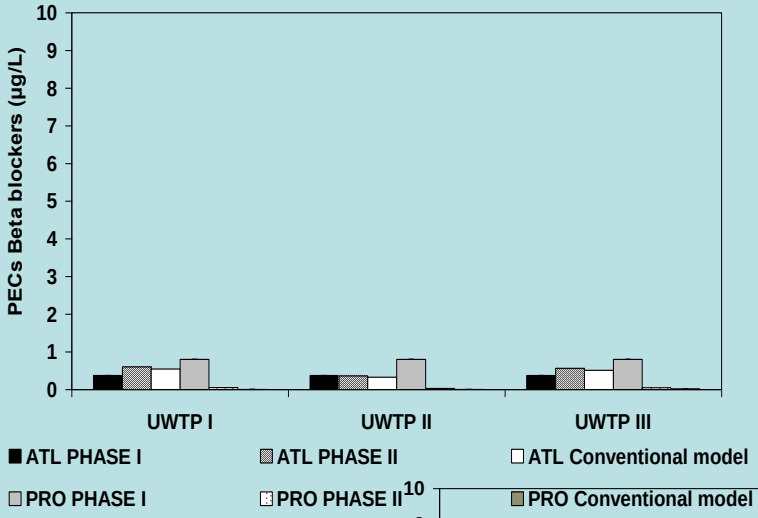
$$PEC_{SURFACEWATER}(\mu g l^{-1}) = \frac{DOSE_{ai} x F_{pen}}{WASTEW_{inhab} x DILUTION x 100}$$

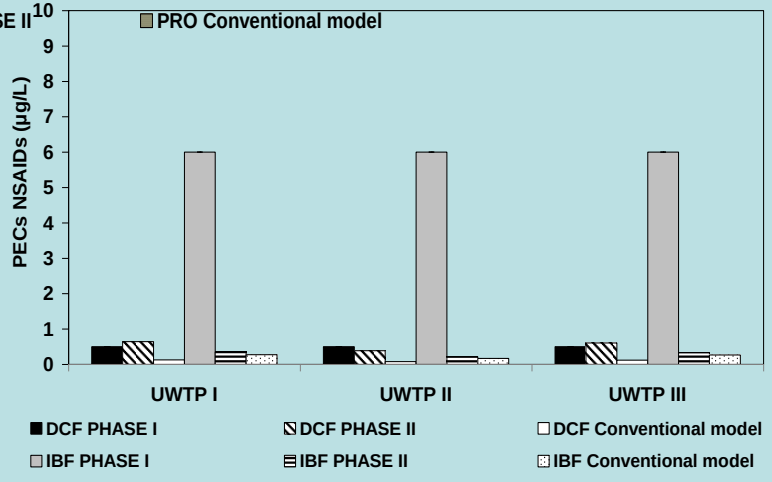
Conventional model

$$PEC(\mu g l^{-1}) = \frac{Ax(100 - R)}{365xPxVxDx100}$$


(A)
PECs AMX (µg/L)
(B)
PECs ERM (µg/L)
(C)
PECs OFX (µg/L)
(D)
PECs SMX (µg/L)
UWTP I
UWTP II
UWTP III
PHASE I
PHASE II
Conventional model


PECs Carbamazepine (µg/L)
UWTP I
UWTP II
UWTP III
PHASE I
PHASE II
Conventional model


PECs Beta blockers (µg/L)
UWTP I
UWTP II
UWTP III
ATL PHASE I
ATL PHASE II
ATL Conventional model
PRO PHASE I
PRO PHASE II
PRO Conventional model


PECs NSAIDs (µg/L)
UWTP I
UWTP II
UWTP III
DCF PHASE I
DCF PHASE II
DCF Conventional model
IBF PHASE I
IBF PHASE II
IBF Conventional model

T2: Analysis, prediction and risk prioritization of active pharmaceutical substances

Regarding the assessment of the environmental risk of pharmaceuticals, acute effects have been reported to be unlikely due to their low environmental concentrations.

As STP effluents have been shown to contain mixtures of pharmaceuticals, their metabolites and transformation products, it is now considered important to study possible combination effects of pharmaceuticals in chronic studies.

- **Composite sampling from 3 points**
  - **Inlet**
  - **Secondary settlement tank**
  - **Outlet after chlorination**

Measured environmental concentrations (MECs), in press !!

- Aegean University, Greece
- Spanish Sientific Council Laboratory, Barcellona

Detected levels of the target analytes in wastewater samples (in μg/L)

UWTP	I			II			III		
Compounds	A	B	C	A	B	C	A	B	C
Ketoprofen	0.34	bld[1]	bld	bld	bld	bld	1.75	0.27	bld
Naproxen	bld	bld	bld	bld	bld	bld	0.21	0.03	bld
Ibuprofen	1.43	0.52	bld	1.31	0.28	0.28	2.20	4.34	3.46
Indomethacine	bld	bld	bld	bld	bld	bld	bld	bld	bld
Diclofenac	0.61	2.11	0.68	2.43	15.41	5.51	0.73	2.99	0.12
Mefenamic acid	bld	bld	bld	bld	bld	bld	bld	bld	bld
Acetaminophen	309.29	0.11	0.07	77.56	0.11	0.07	405.37	0.05	0.10
Propyphenazone	bld	0.04	0.03	bld	bld	bld	bld	bld	bld
Clofibric acid	bld	bld	bld	0.00	0.00	0.00	bld	bld	bld
Gemfibrozil	bld	0.00	bld	0.00	0.00	0.00	bld	bld	bld
Bezafibrate	0.51	0.14	0.05	0.73	0.29	0.22	0.99	0.14	0.11
Pravastatin	bld	bld	bld	bld	bld	bld	bld	bld	bld
Mevastatin	bld	bld	bld	bld	bld	bld	bld	bld	bld
Carbamazepine	0.76	0.84	0.57	14.45	24.54	27.27	2.61	1.49	1.38
Fluoxetine	bld	bld	bld	bld	bld	bld	bld	bld	bld
Paroxetine	bld	bld	bld	bld	bld	bld	bld	bld	bld
Lansoprazole	bld	bld	bld	bld	bld	bld	bld	bld	bld
Loratadine	bld	bld	bld	bld	bld	bld	bld	bld	bld
Famotidine	1.00	0.59	bld	2.78	5.06	bld	1.30	0.38	bld
Ranitidine	0.07	0.08	bld	0.14	0.47	bld	0.43	0.31	bld
Erythromycin	0.38	0.20	0.03	0.28	0.25	0.40	0.70	0.42	bld
Azythromycin	1.15	1.60	0.18	0.66	0.30	0.20	1.68	0.53	0.03
Sulfamethoxazole	1.07	0.19	0.01	1.51	0.78	0.46	5.41	0.64	0.03
Trimethoprim	0.05	bld	bld	0.14	0.09	blq[2]	0.35	0.06	bld
Ofloxacin	22.62	3.02	1.29	34.74	5.93	4.82	59.38	3.33	1.90
Atenolol	3.29	0.12	0.13	3.15	0.89	0.73	5.81	0.92	0.94
Sotalol	2.81	0.10	0.11	2.70	0.76	0.62	4.97	0.79	0.81
Metoprolol	1.30	0.98	0.57	2.09	1.23	9.59	1.49	1.31	0.69
Propranolol	0.27	0.49	blq	0.41	0.59	0.28	0.23	0.44	blq

[1]bld: below limit of detection
[2]blq: below limit of quantitation
A: inlet, B: after secondary treatment, C: outlet

T3: Development of methods for assessing WET and mixture toxicity in synthetic samples

- **Organisms to be used for bioassaying**
  - *Selenastrum capricornutum* *
  - *Daphnia magna*
  - *Ceriodaphnia dubia*
  - *Artemia salina*
  - *Dunaniella tertiolecta*
  - *Vibrio fischeri*
  - Sea urchins
  - Plants →
  - *YES* →

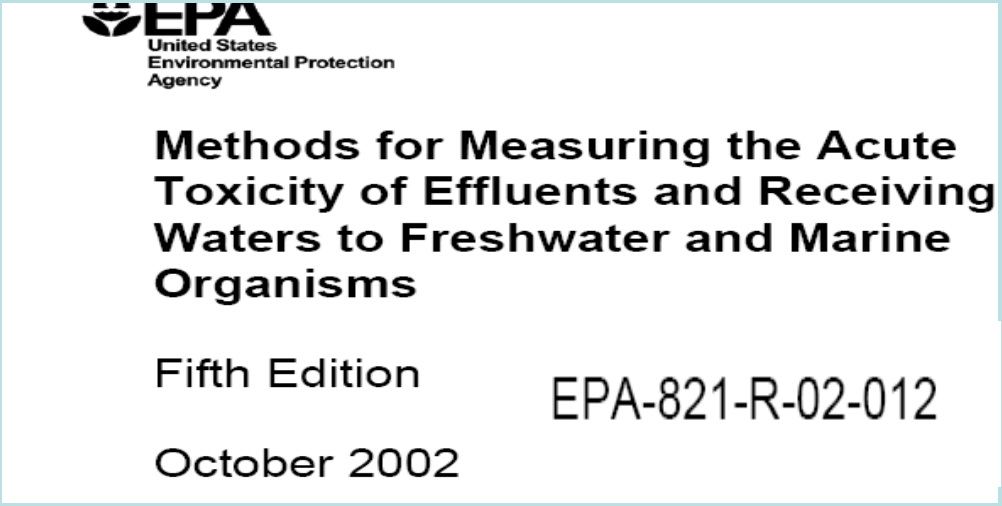
Freshwater

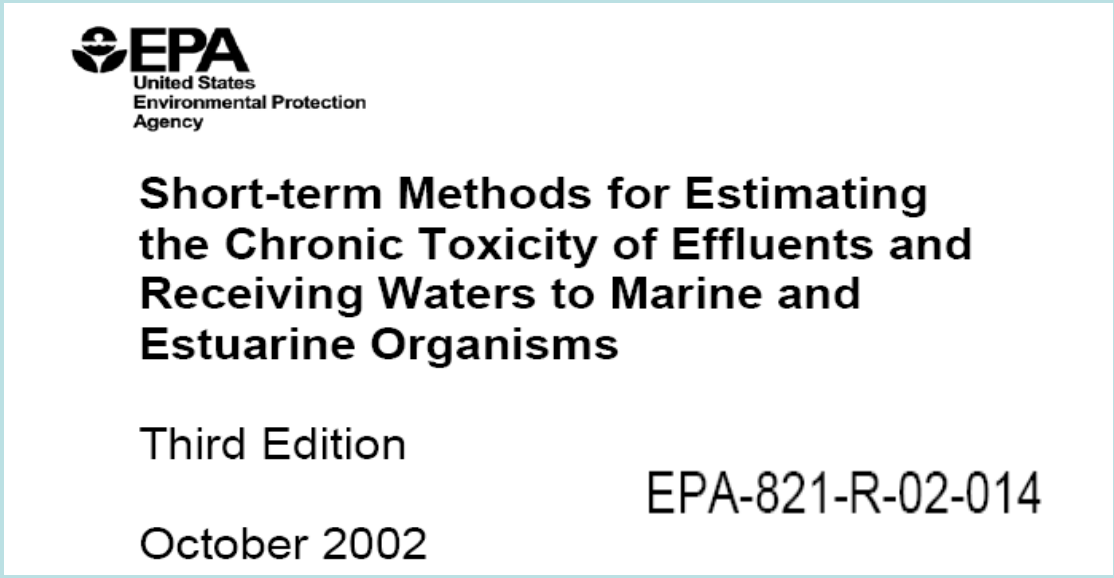
Saline water

Terrestrial plant seeds

*** The methods have been used to test the samples till present**

WET approach





WET approach

- Whole effluent toxicity (WET): the aggregate toxic effect of an effluent measured directly by an aquatic toxicity test. [54 FR 23686; June 2, 1989].

•Exposure durations:

•generally 24 hours (acute tests)-7 days or more (short-term chronic or chronic tests).

Biological endpoints:

survival, growth, reproduction, or fertilization.

Effect concentrations:

NOEC (No Observed Effect Concentration),

LOEC (Lowest Observed Effect Concentration),

LC_{50} (median lethal effect concentration),

EC_{50} (median effect concentration), or IC25 (25% inhibition concentration)

are commonly used to report the results of WET tests.

Sampling transfer & analyses

- Composite samples were transfered in cooled boxes in glass containers to the lab and kept at +4 ^{0}C till transfering to Italy (2 days).

- The collected samples were delivered by air-transfer (1 day) in cooled bags (+4 ^{O}C) to toxicology laboratory (ERL-UNINA). They were kept refrigerated at +4 ^{O}C during toxicity tests (one week).

- Conventional parameters were measured at the Cyprus lab.

- Samples were checked for D.O and free chlorine, and when needed, Free Cl_2 was blocked by thyopentasulphate.

ERL-UNINA

Ecotoxicology Research Laboratory

(*www.erl.unina.it*)

Xenobiotics

- -Pesticides
- -Pharmaceuticals
- -*Plantothrix* (Cytotoxins)
- -Surfactants
- -Antifouling-Biocides
- -Disinfection by-products
- -Flame-retardants
- -Metals
- -Personal care products
- -Nanoparticles

Testing methods

- *-Artemia salina*
- *-Daphnia magna*
- *-Selenastrum capricornutum*
- *-Vibrio fisheri*
- *-Ceriodaphnia dubia*
- *-Dunaniella tertiolecta*
- *-Lepidium sativum*
- -Mutox, gentox
- -Fish
- -Sea urchins
- -Inhibition-BOD tests
- -ELISA

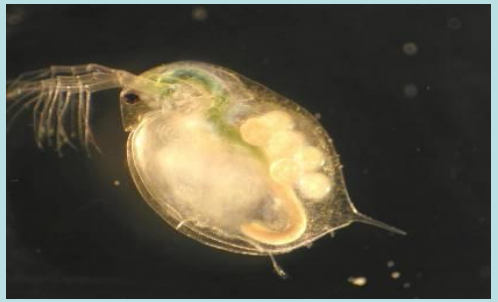
Artemia salina

- *Artemia* cysts (RAC) (AF/N2000) were obtained from the Quality Assurance Research Division, USEPA, Cincinnati, OH, USA and the Laboratory for Biological Research in Aquatic Pollution, University of Ghent, Belgium by the certification of *A. franciscana* in Italy (ECOTOX srl).
- The cysts were activated in a standard marine solution (35‰ salinity, Ocean ®).
- *20 A. franciscana* nauplii (<48-h old) were exposed to fungicides solutions for 24 h as 4 replicated in 2 mL samples for 24 and 48 h.
- Both negative control with Ocean ® and positive control with $K_2Cr_0O_7$ tests were performed in parallel.

% Immobilization: (number of tested organisms- number of immobilized organisms) *100 / no of tested organisms

Daphnia magna

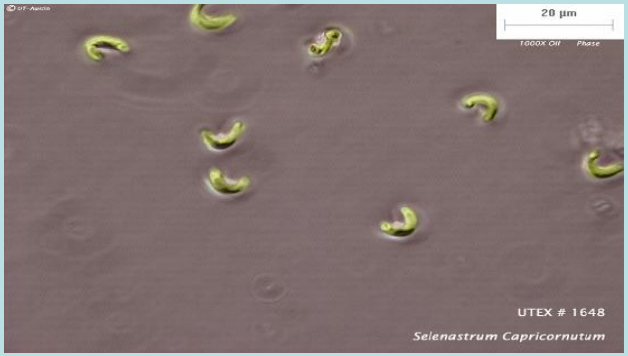
- Newborn daphnids (<24-h old) were exposed to fungicides solutions for 24 h.
- Daphnids were grown at 20°C ± 1°C under light conditions of 4,000 lux using cool light lamps and tested at the same temperature without light emission. They were fed with *S. capricornutum* (300,000 cells/mL) and baker's yeast (*Schizosaccharomyces cerevisiae*, 200,000 cells/mL).
- Tests were conducted in quadruplicate using 5 daphnids in eack beaker.
- 20 daphnids were scored for their frequencies of immobilization.
- Negative and positive tests were performed in parallel to fungicides testing.



% Immobilization: (no of tested organisms- no of immobilized organisms)*100 / no of tested organisms

Selenastrum capricornutum

- Unicellular freshwater green microalgae from the species *Selenastrum capricornutum* were used.
- Weekly transplanted algae in exponential growth were cultured.
- Cultures were kept in Erlenmeyer flasks at the same conditions with *Daphnia magna.*
- The toxicity tests were initiated from an algal concentration of 3,000 cells/mL and conducted in nine replicates.
- Negative and positive control tests were performed in parallel
- The endpoint consisted of percent algal growth, which was measured after 96 h in a Bürker cell counting chamber.



% cell growth inhibition: (cell number in blank- cell number in the sample)*100 / cell number in blank

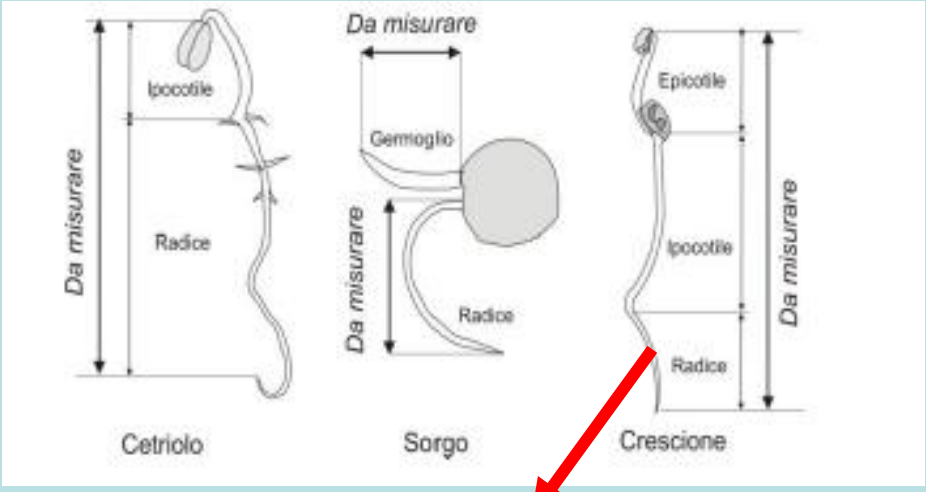
Lepidium sativium

L. sativum L. seeds were obtained from F.lli Ingegnoli
(Milano, Italy)

The *L. sativum* seeds were germinated in disposable Petri dishes, (100mm in diameter), on Whatmann filter paper moistened with 5mL
of either double-distilled (dd) water (control)
.

Tests were run in triplicate, on 10 seeds per dish. Petri dishes were kept in the dark, at 25 ◦C, for 72 h.

The length of the whole plantlet and of root was measured with a ruler (against a black background.



End points: Inhibition of Germination and Rooth lenght

Index of inhibition versus blank (IG %)
%IG = (G1L1)/(GcLc) *100

where G1: germinated seed number exposed to sample, and Gc: germinated seed number exposed to negative control medium, L1: length of roots exposed to to sample and Lc: length of root exposed to negative control medium..

A multi-species whole effluent toxicity (WET) testing approach for urban wastewater treatment plants, to be submitted !!

Despo Fatta-Kassinos[1], Marlen I. Vasquez[1], Marco Guida[2], Sureyya Meric[2]*

WET (100% tested) approach results of the tested samples *vs* used bioassays

Bioassays	Sample points	UWTP I	UWTP II	UWTP III
P. subcapitata	Influent	**T**	**T**	**T**
	Effluent	M	**T**	**T**
AlgalTox kit	Influent	**T**	**T**	**T**
	Effluent	**M**	**T**	**T**
D. magna	Influent	**T**	**T**	**T**
	Effluent	L	**T**	**T**
DaphTox kit	Influent	T	T	T
	Effluent	T	T	T
V. fischeri	Influent	M	M	M
	Effluent	ST	ST	M
A. salina	Influent	ST	ST	**T**
	Effluent	ST	ST	ST
A. salina (Larnaca mulsh)	Influent	T	T	T
	Effluent	ST	ST	ST
L. sativum	Influent	ST	ST	ST
	Effluent	ST	ST	ST
C. sativus	Effluent	**H**	ST	**H**
S. saccaratum	Effluent	ST	**H**	**H**

Toxic (T): 75-100% toxicity;

Moderate (M): 50-75% toxicity;

Slight toxic (ST): <50% toxicity;

Hormesis >100% stimulation;

Testing of pharmaceuticals single and in the mixtures

- There are few studies available in the literature on mixture toxicity of pharmaceuticals, and some examples are:

- ibuprofen,
- fluoxetine,
- ciprofloxacin

to *Lemna gibba* and *Myriophyllum* spp for 35 d (Richards et al. (2004);

- atorvastatin,
- acetaminophen,
- caffeine,
- sulfamethoxazole,
- carbamazepine,
- levofloxacin,
- sertraline,
- trimethoprim

a variety of somatic and pigment endpoints in rooted (*M. sibiricum*) and floating (*L. gibba*) (Brain et al., 2004);

- b1-selective blockers

(acebutolol, atenolol, and metoprolol)

- non-b1-selective blockers

(nadolol, oxprenolol, and propranolol)

in mixture using acute 2 d *Ceriodaphnia dubia* immobility test (Fraysse and Garric, 2005);

- acetaminophen,
- diclofenac,
- gemfibrozil,
- ibuprofen,
- naproxen,
- salicylic acid,
- Triclosan

freshwater amphipod *Hyalella azteca* over three generations (Borgmann et al., 2007).

- A: Carbamazepine, clofibric acid,
- B: diclofenac and ibuprofen
- C: diclofenac, ibuprofen, naproxen and acetylsalicylic acid
- D: b-blockers (propranolol, atenolol, metoprolol).

Cleuvers (2003; 2004; 2005)
Daphnia magna
Lemna minor
Desmodesmus subspicatus

Study design

For each mixture three concentrations were tested by adding from each of those nine compounds as 0.25, 0.5 and 1 mg/L. These concentrations were chosen due to the reasons of:

1) Webb (2001) reported, in a review, over 360 acute measured endpoints for 107 pharmaceuticals, that over 90% were at concentrations superior to 1 mg/L, suggesting the relative limited acute ecotoxicity of pharmaceuticals: what about mixture compounds!!

2) As noted in the literature findings that a range (form realistic (low) to effective (high)) of concentrations screening was needed to examine the behaviour of the pharmaceuticals in the mixture (Brain et al., 2004; Richards et al., 2004; Fraysse and Garric, 2005; Borgmann et al., 2007).

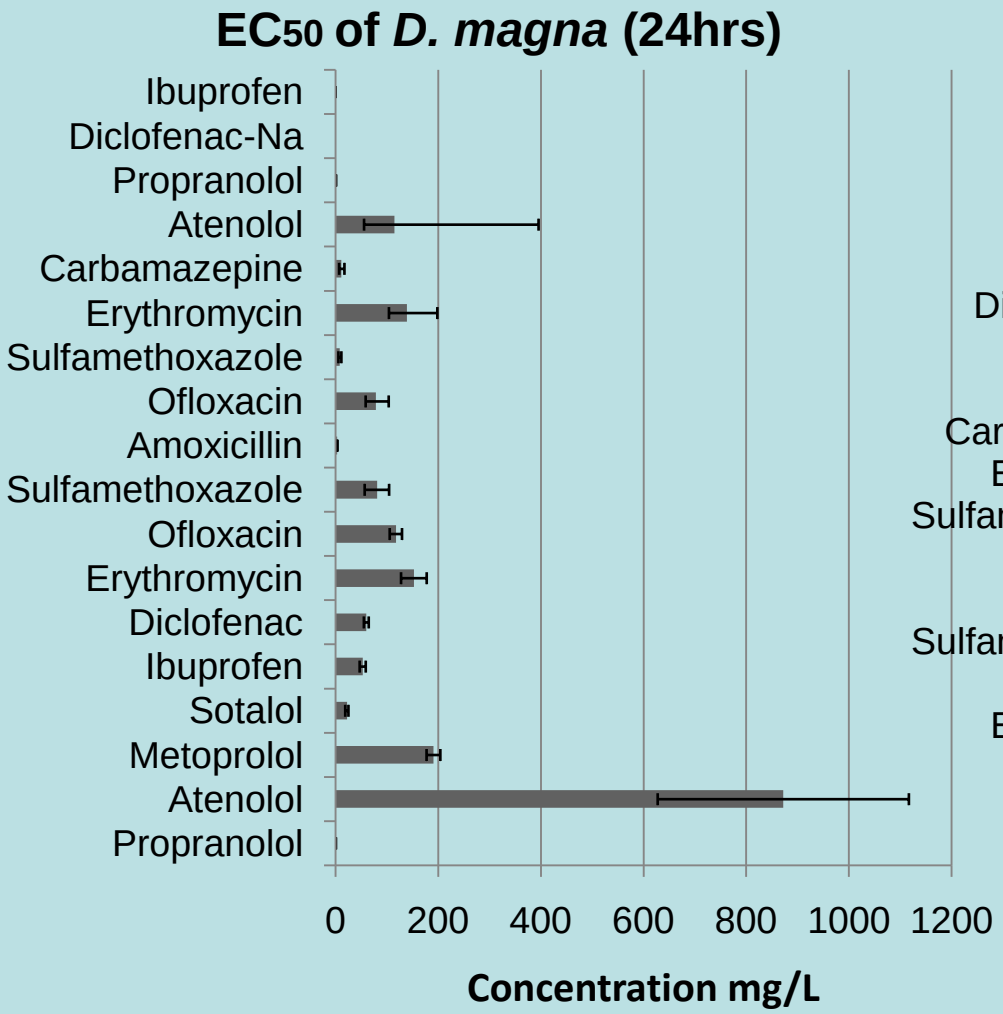
3) The envrionmental concentrations of pharmaceuticals (MECs) have been detected in UWTP effluents varying from ng to µg/L concentrations and their risk assessment procedure starts from 10 ng/L (Bound and Voulvoulis, 2004; EMEA; 2006).

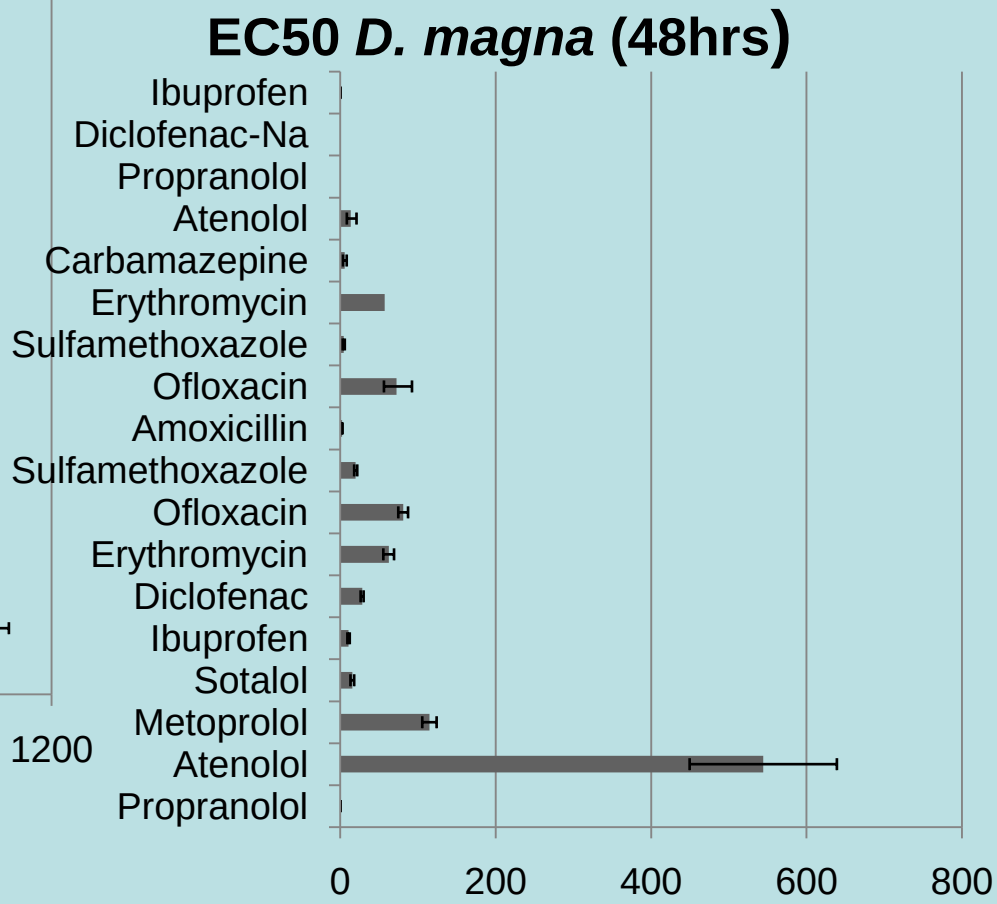
Mixture no	ATL	PRP	DCF	IBF	CBZ	OFL	SMX	ERY	AMX
1									
2									
3									
4									
5									
6									
7									
8									

ATL (Atenolol); PRP (Propranolol); DCF (Diclofenac); IBF (Ibuprofen); CBZ (Carbamazepine); OFL (Ofloxacin); SMX (Sulfamethoxazole); ERY (Erthromycin); AMX (Amoxicillin)

Single and mixture toxicity results

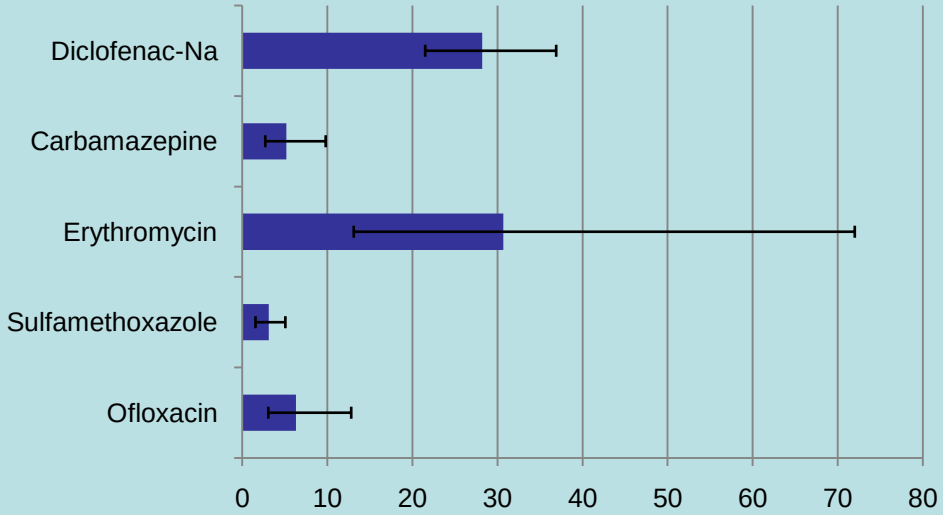
Single compunds



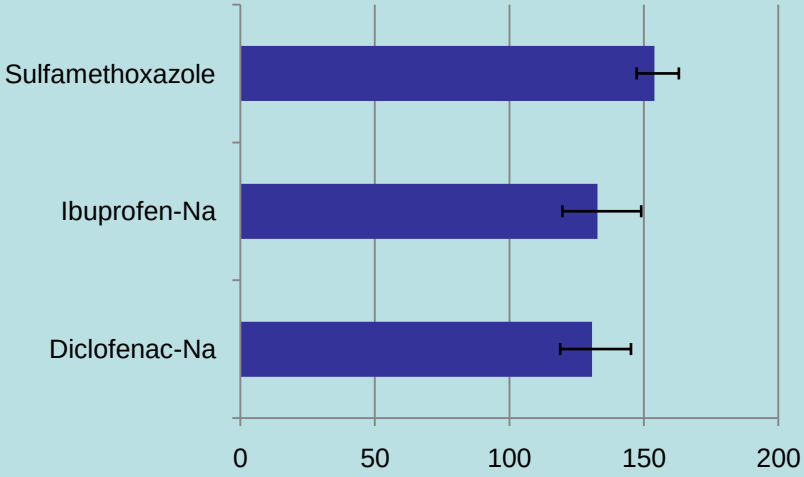


Single compounds

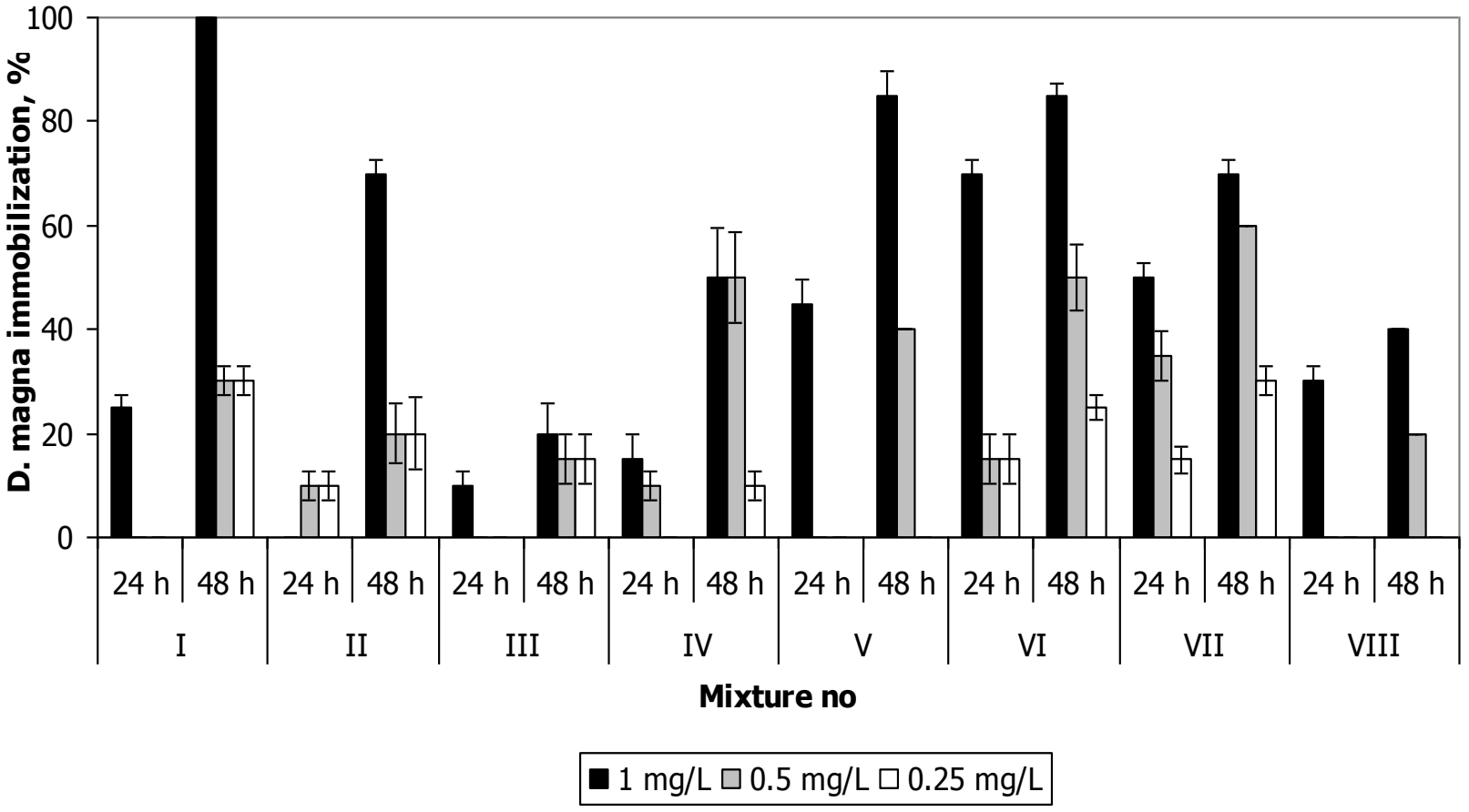
***P. subcapitata*, EC50 (96hrs)**



***D. tertiolecta*, EC50 (96hrs)**



Mixtures-Daphnia magna



	Artemia salina, mean % immobilization															
Mixture no.	**I**		**II**		**III**		**IV**		**V**		**VI**		**VII**		**VIII**	
Exposure time	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
1 mg/L	10	15	0	0	0	5	0	10	15	15	0	0	0	0	0	0
0.5 mg/L	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25 mg/L	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	P. subcapitata, 96 h							
Mixture no	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
1 mg/L	100	98	100	96	68	50	65.6	57.88
0.5 mg/L	96	86	98	90	50	-118	40.625	52.63
0.25 mg/L	93	84	88	93	25	53	37.5	31.58

	L. sativum Germination index %, 72 h							
Mixture no	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
1 mg/L	111.3	104.7	97.13	82.27	84.378	55	27.99	24.696
0.5 mg/L	113.2	122	105.1	89.72	84.958	47	27.96	49.79
0.25 mg/L	141	123.6	123.3	112.5	111.15	96.85	18	57.387

- Conclusion
- Toxicity is varying among the groups of the pharmaceuticals that the relationship '*species-response'* is more significant than the '*dose-response'* one.

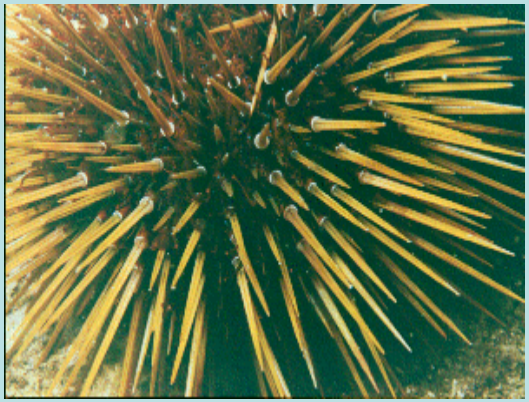
- *P. subcapitata* was the most sensitive species for all the mixtures tested. That of *D. magna* followed this species's sensitivity.

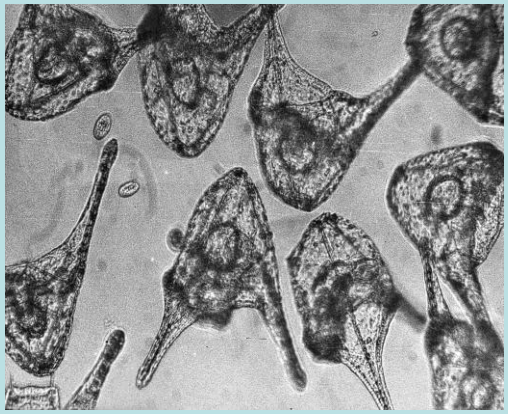
- The model evaluation of our results presented hereby did not obey either to CA or Dissimilar action *(DA)* model (*antagonism)*. This can be due to
- i) the EC_{50} values obtained in this study may need to be re-evaluated using other models (e.g. Linear, Logistic, Gompertz, Exponential, Hormetic models) to fit the concentration–response relationships;
- ii) any risk model beyond CA and DA models which is an undergoing study in parallel ;
- iii) increasing model species in order to obtain accurate data sets for testing appropriate models.

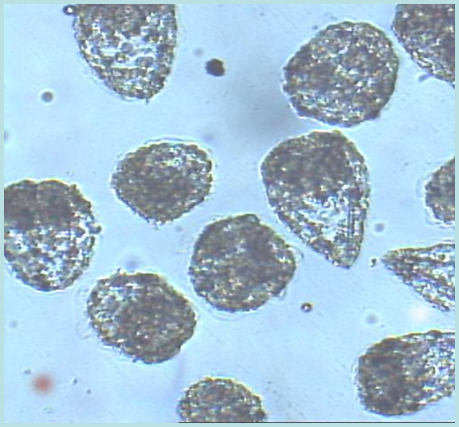
Current work

Cell line

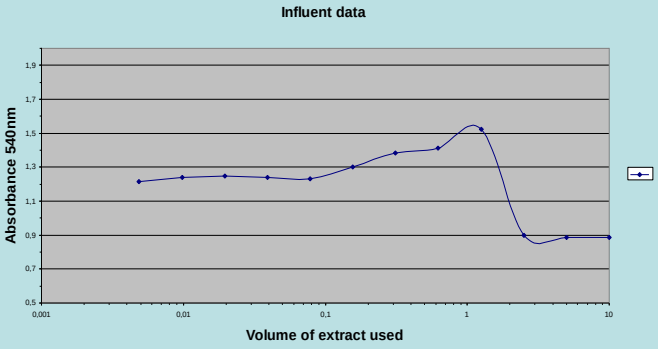
Sea urchnis-emrbryotoxicity, spermitoxicity, cytotoxicity, devlopmental toxicity

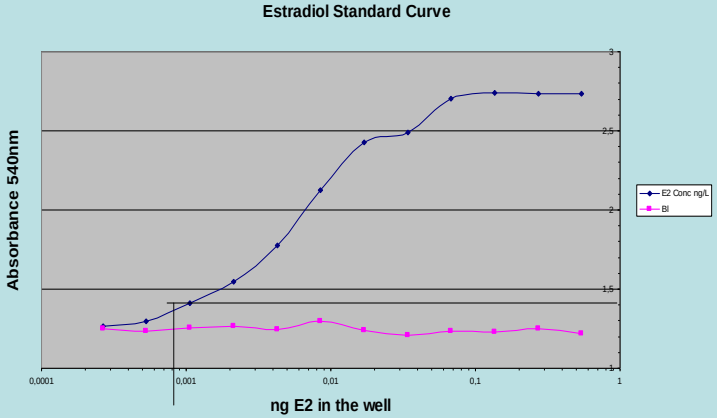






Estogenicity

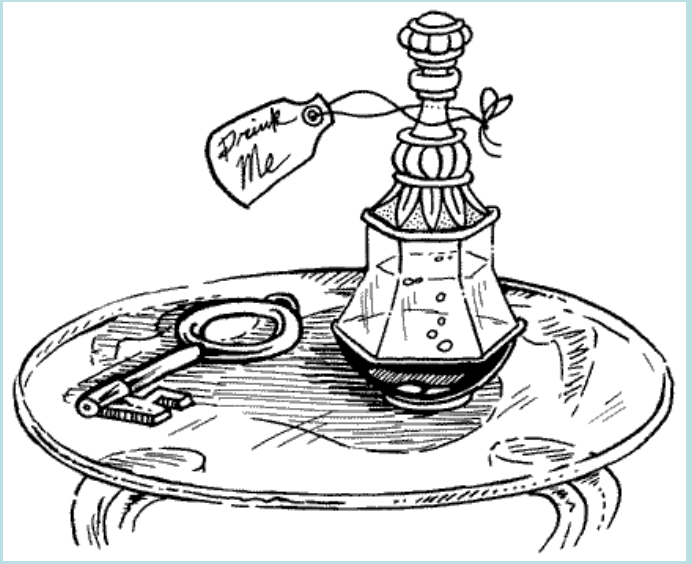




Drink .....

'...*and she had never forgotten that, if you drink much from a bottle marked "poison" it is almost certain to disagree with you, sooner or later'.*

From Alice's Adventures in Wonderland. Lewis Carroll



Thank you for your kind attention

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