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ON SUSTAINABLE ENVIRONMENT AND SEAS

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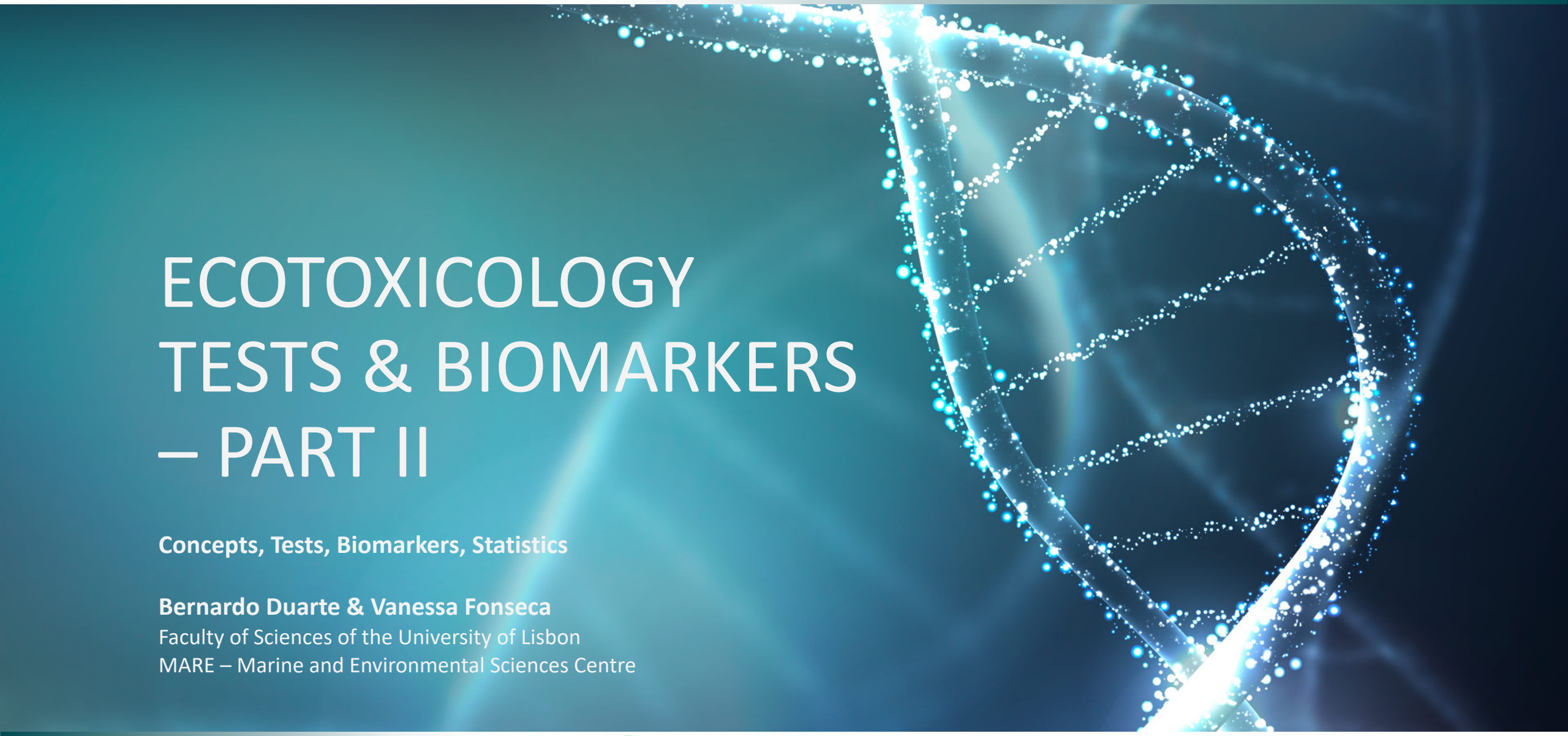

ECOTOXICOLOGY TESTS & BIOMARKERS – PART II

Concepts, Tests, Biomarkers, Statistics

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MARE – Marine and Environmental Sciences Centre

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BIOMARKERS

Oxidative stress, ROS, biochemical reactions, enzymes, anti-oxidant, damage biomarkers

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BIOMARKERS : INTRODUCTION

BIOINDICATOR

Organisms that express specific symptoms or responses that indicate environmental changes. Produces **QUALITATIVE** information regarding these changes (better, worse than a previous or reference condition).

BIOMONITOR

Organisms or populations whose distribution is studied over time and space and compared to a model where the deviations to the expected behaviour are evaluated. Produce **QUANTITATIVE** information regarding the environmental changes.

BIOMARKER

A trait or molecular entity that can be measured experimentally and indicate the occurrence of a certain function (normal or pathological) of a certain organisms towards a specific stressor. Ideally these biomarkers should produce a dose related response towards the stressor applied.

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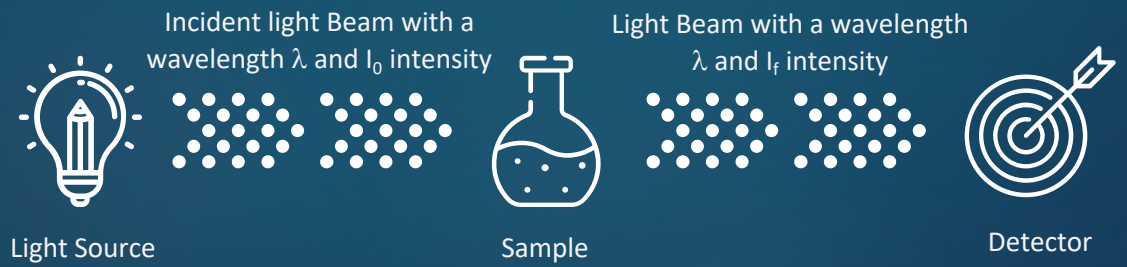
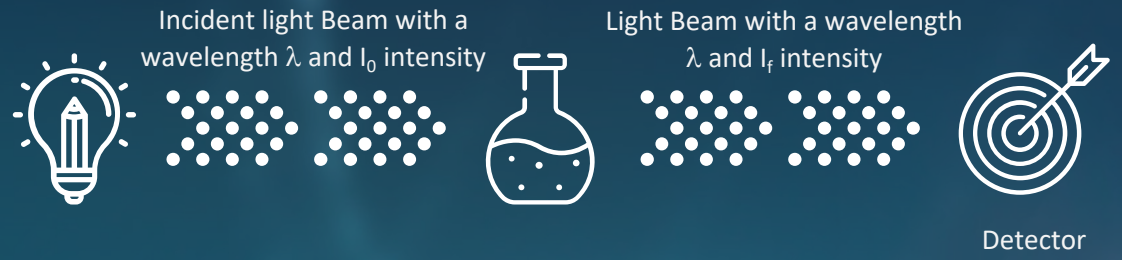
SPECTROPHOTOMETRY APPLICATIONS IN ECOTOXICOLOGY:

- Spectrophotometry is a tool that hinges on the quantitative analysis of molecules depending on how much light is absorbed by colored compounds. Spectrophotometry uses photometers, known as spectrophotometers, that can measure a light beam's intensity as a function of its color (wavelength). Important features of spectrophotometers are spectral bandwidth (the range of colors it can transmit through the test sample), the percentage of sample-transmission, the logarithmic range of sample-absorption, and sometimes a percentage of reflectance measurement.
- Most biomarkers can be evaluated using spectrophotometric assays.



Absorbance (Abs) is the amount of light absorbed by the sample

$Abs = I_0 - I_f$



Absorbance (Abs) is the amount of light absorbed by the sample
Abs = $I_0 - I_f$, with $I_0 > I_f$

LAMBERT-BEER LAW:

- All spectrophotometric analysis follow the Lambert-Beer Law.
- This law relates the attenuation of light to the properties of the material through which the light is travelling and describes the relationship between the absorbance of a certain compound with its concentration.

$Abs_\lambda = [Compound\ A] \times L \times \varepsilon_\lambda$

[Compound A] – molar concentration of the compound in analysis

L – light path through the sample (typically in most cuvettes is 1 cm so this term can be nullified from the equation)

ε_λ – Molar extinction coefficient of the compound at a wavelength of λ nm

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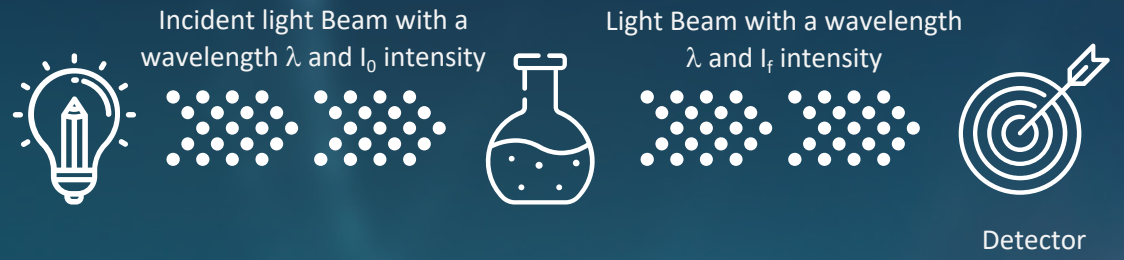

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ε_λ – Molar extinction coefficient of the compound at a wavelength of λ nm.

$Abs_\lambda = [Compound\ A] \times L \times \varepsilon_\lambda$, if L = 1 cm $\Leftrightarrow$ $Abs_\lambda = [Compound\ A] \times \varepsilon_\lambda$,

This equation resembles a linear correlation equation from the type of y = mx + b

$y = Abs_\lambda$ (dimensionless)

$m = \varepsilon_\lambda$ (units: $M^{-1}\ cm^{-1}$)

x = [Compound A] (units: M)

Considering that at [Compound A] = 0 then $Abs_\lambda = 0$ then b = 0

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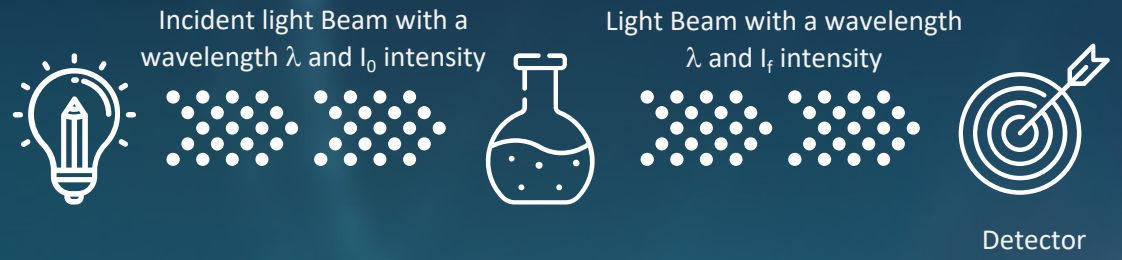

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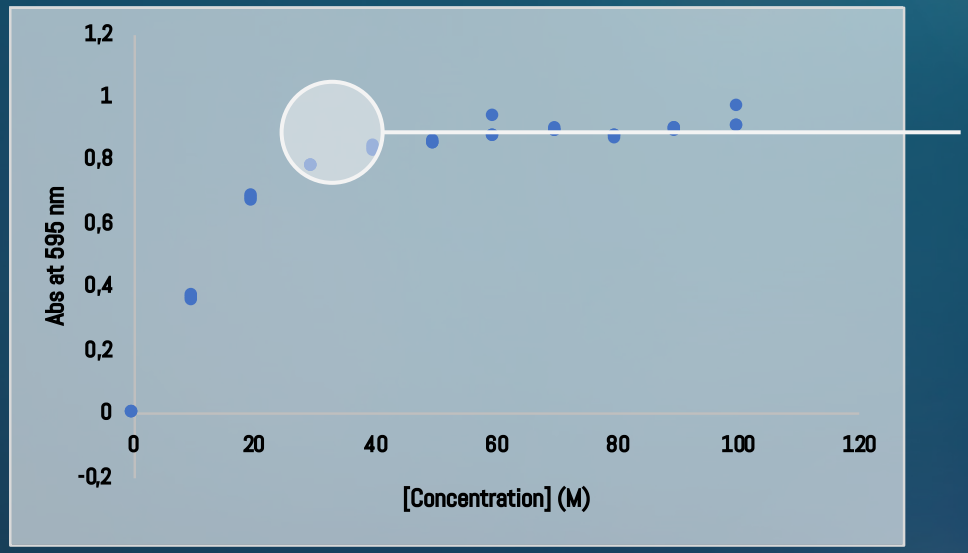
This can be attained with a calibration curve were several known concentration standards are analysed for its absorbance and a calibration curve is established.



UPPER DETECTION LIMIT (Abs=0.781)

At this point the Lambert-Beer law does not apply any more, as the concentration continues to increase but the absorbance doesn't follow this trend

Samples with absorbance above this value should be diluted in order to have an absorbance within the linear range of the calibration curve

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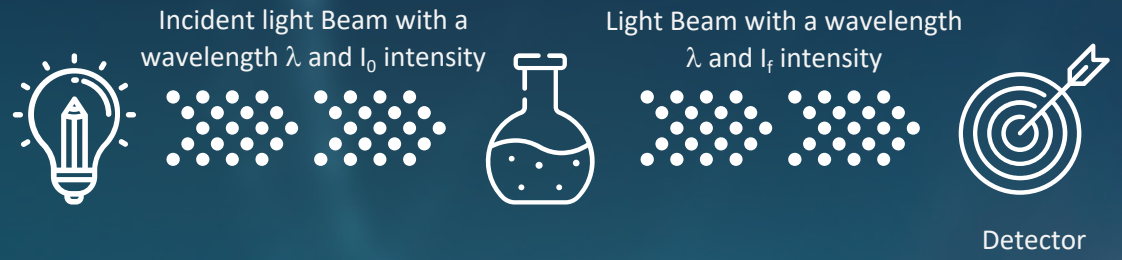
BIOMARKERS : SPECTROPHOTOMETRY



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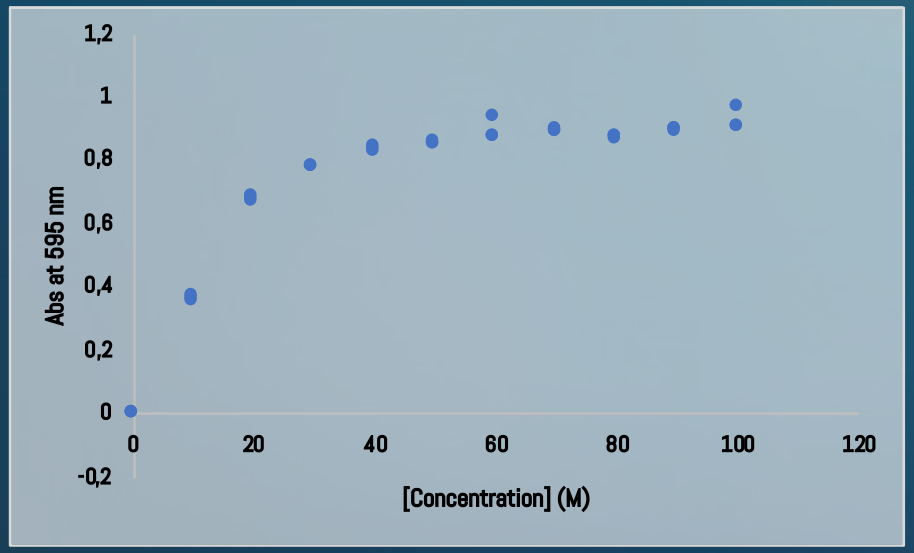

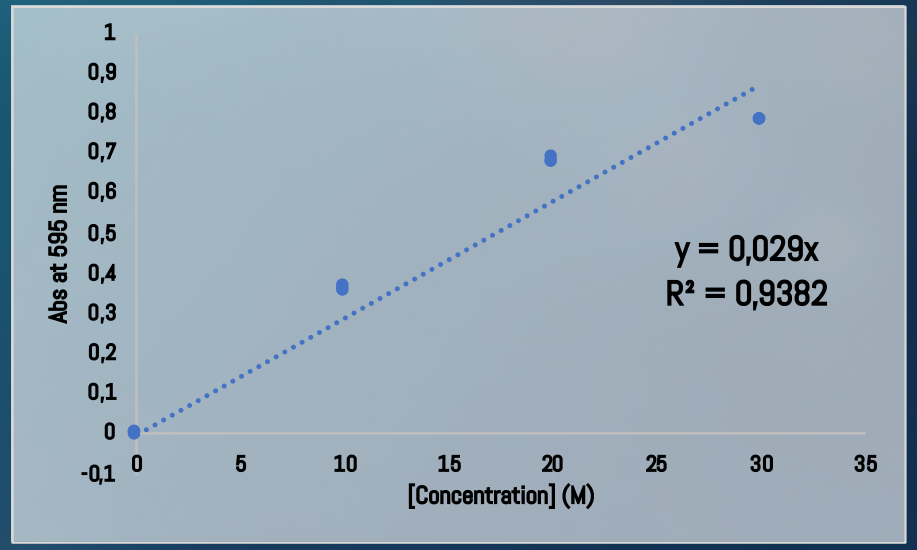

Abs (y) = ε_λ x [Concentration]

$\varepsilon_\lambda = 0.029\ M^{-1}\ cm^{-1}$

Using this equation any sample can be analysed for its absorbance and its concentration derived from th

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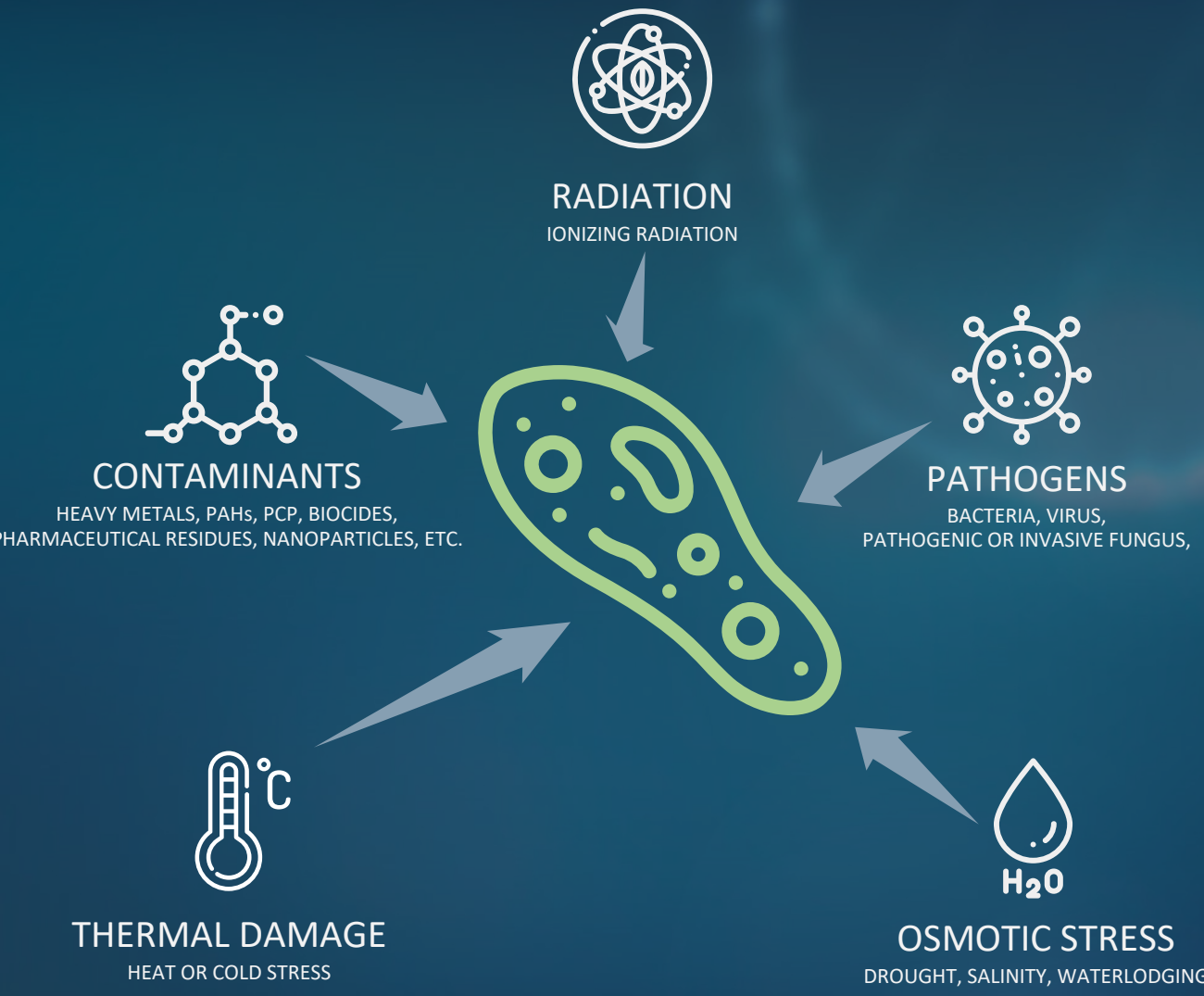
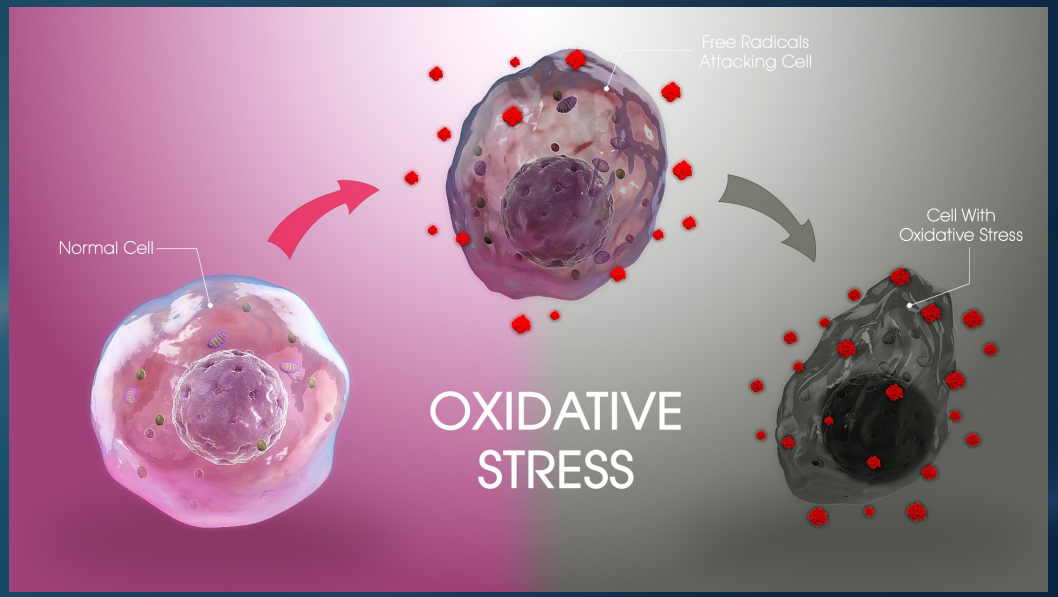
BIOMARKERS : OXIDATIVE STRESS

OXIDATIVE STRESS:

- Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.
- Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.
- Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. Base damage is mostly indirect and caused by reactive oxygen species (ROS) generated, e.g. O^{2-} (superoxide radical), OH (hydroxyl radical) and H_2O_2 (hydrogen peroxide).
- Furthermore, some reactive oxidative species act as cellular messengers in redox signalling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signalling.
- IMPORTANT: ROS are generated during the normal cell functioning thus the cells also developed anti-oxidant mechanisms to counteract the negative effects produced by ROS.
- In Ecotoxicology, the production or activity of these ROS counteractive measures as well as the ROS-biomolecules reaction products are used to evaluate the cell oxidative stress level.

BIOMARKERS : SOURCES OF OXIDATIVE STRESS





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PHASE I: BIOTRANSFORMATION

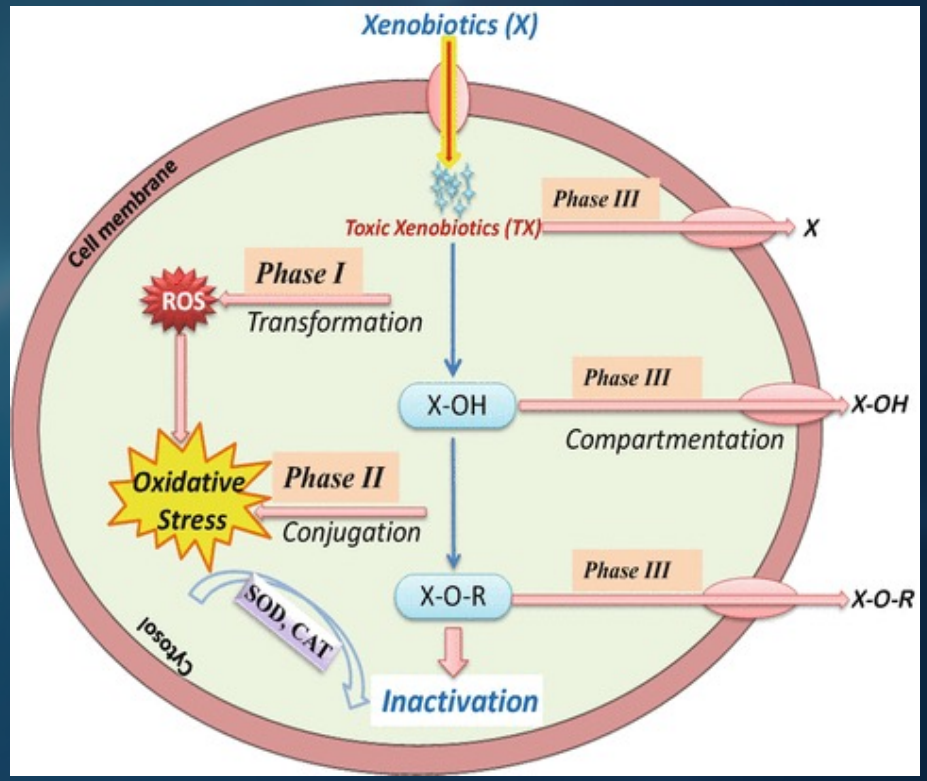
Phase I enzymes and mechanisms transform xenobiotics into less harmful molecules, but that may have a ROS-generating potential (for e.g.: CYPA1 enzymes).

PHASE II: CONJUGATION

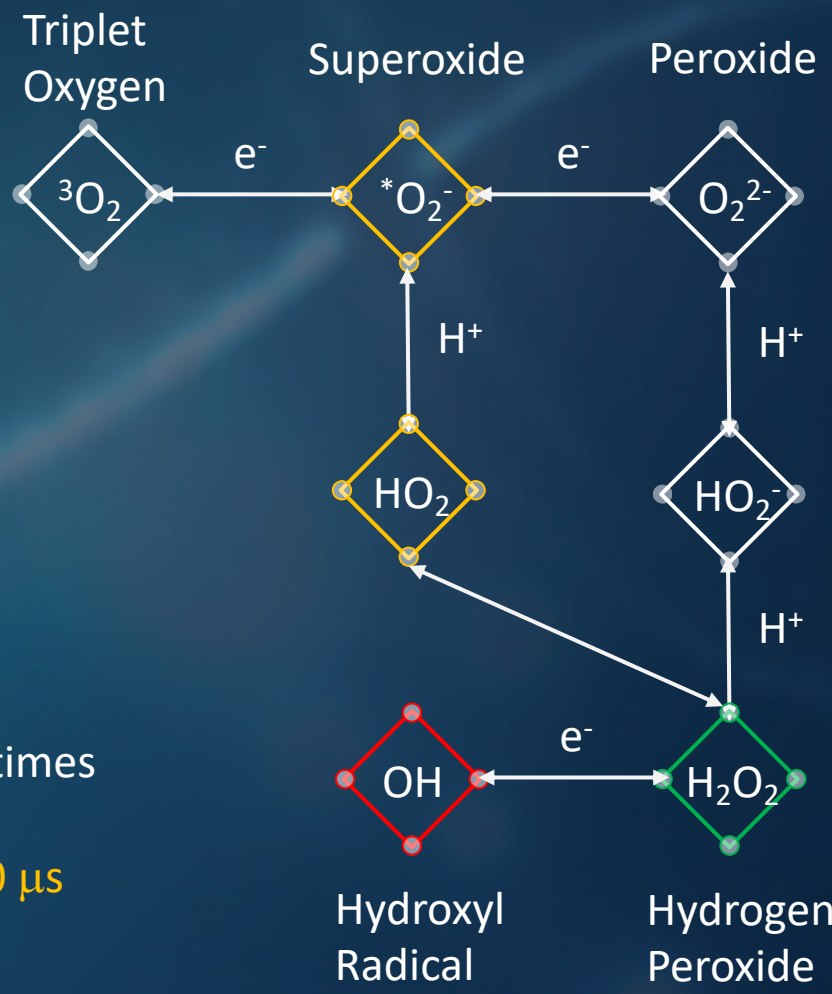
Phase II mechanisms are composed by enzymatic and non-enzymatic anti-oxidant mechanisms that work in conjugation to quench the ROS generated directly by the xenobiotic or by the Phase I biotransformed xenobiotic.

PHASE III: EXCRETION

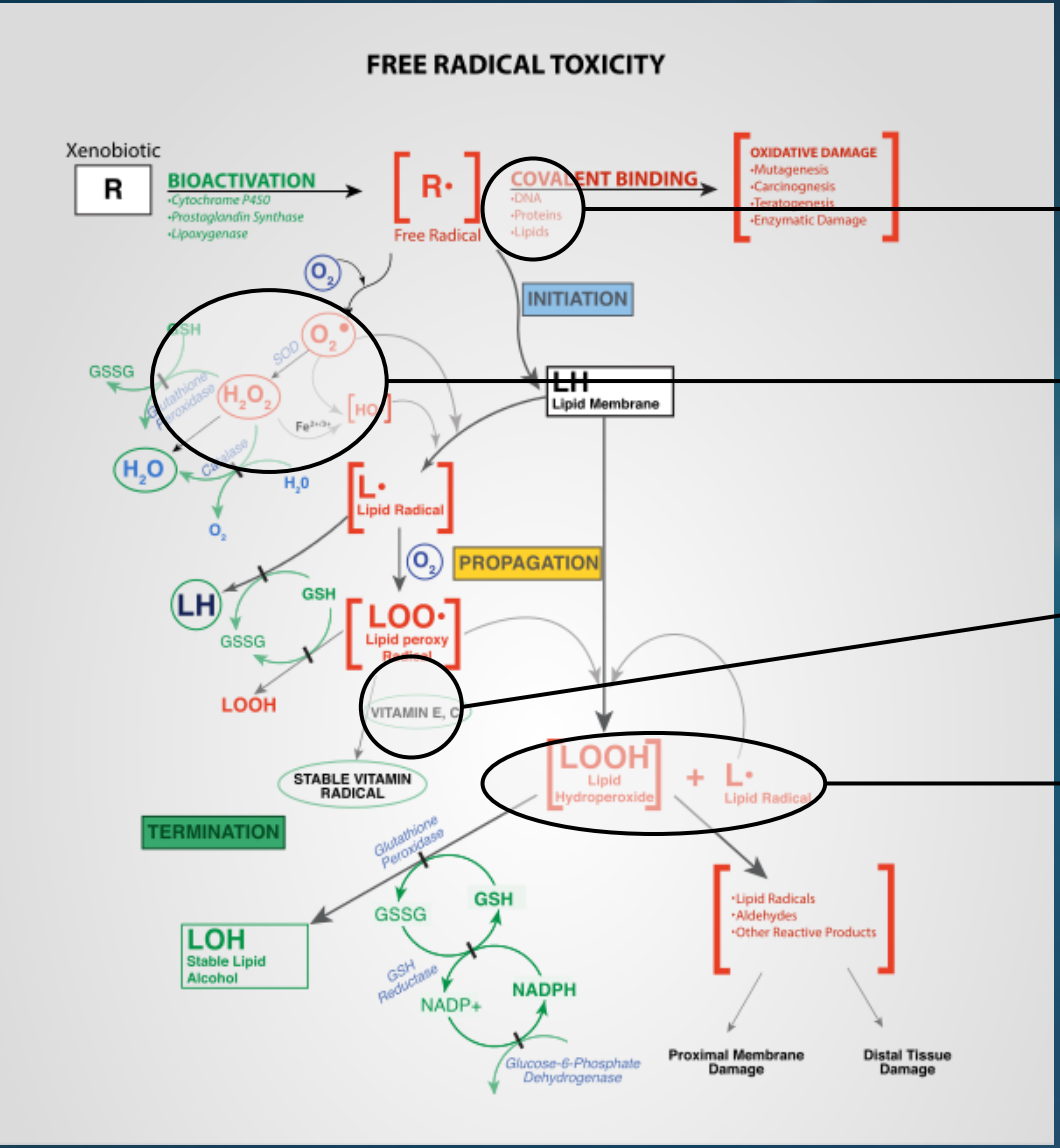
Phase III mechanisms are based in membrane proteins that can excrete directly or throughout vesicle compartments the transformed and/or inactivated xenobiotic to the extracellular environment.



Oxygen	O_2
Superoxide anion	O_2^-
Peroxide anion	O_2^{2-}
Hydrogen peroxide	H_2O_2
Hydroxyl radical	OH
Hydroxyl anion	OH^-
Singlet Oxygen	$^{-1}O_2$
Hypochloric acid	HOCl



ROS lifetimes
1 ns
100 – 10 μs
s – min



DNA AND PROTEIN DAMAGE

ROS covalent binding to DNA leads to double strand disruption.
ROS interaction with Proteins induces protein oxidation/carboxylation.

ENZYMATIC DEFENSE SYSTEM

Superoxide dismutase isoforms and several peroxidases decompose ROS into harmless substances.

NON-ENZYMATIC ANTIOXIDANT

Vitamins, phenolics, flavonoids, thiol molecules and other anti-oxidant are able to quench directly ROS molecules into stable harmless radicals.

MEMBRANE DAMAGE

ROS interaction membrane fatty acids induces the formation of lipid hydroperoxides and lipid radicals inducing membrane disruption.

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BIOMARKERS : TYPES

BIOMARKER

A trait or molecular entity that can be measured experimentally and indicate the occurrence of a certain function (normal or pathological) of a certain organisms towards a specific stressor. Ideally these biomarkers should produce a dose related response towards the stressor applied.

ENZYMES

Typically oxidative stress triggered enzymes, which the activity is related to the stress level imposed.

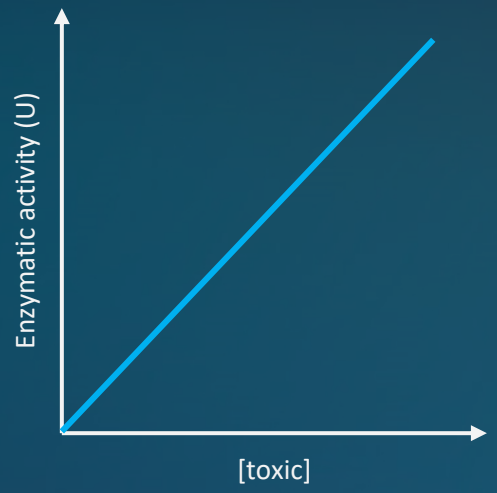
DEGRADATION PRODUCTS

Molecules or compounds that result from the destruction of a cellular structure or metabolite in reaction to stress.

NON-ENZYMATIC ANTI-OXIDANT MOLECULES

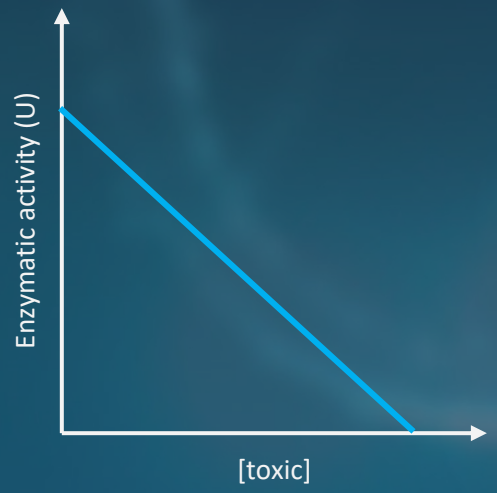
Molecules or compounds that are produced in reaction to oxidative stress.

BIOMARKERS : ENZYMES UNDER STRESS



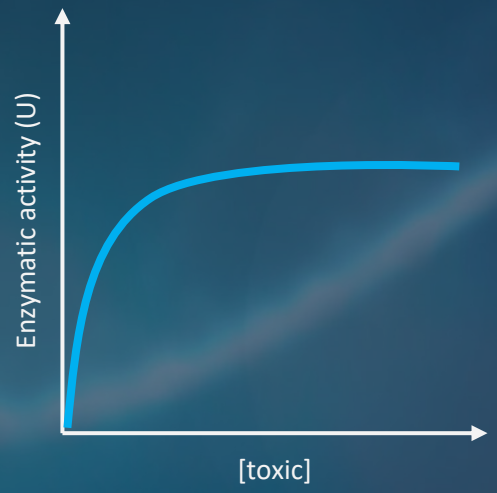
Dose-ACTIVITY PORPOTION

This would be the perfect biomarker. Its activity is proportional to the concentration of the toxic substance.



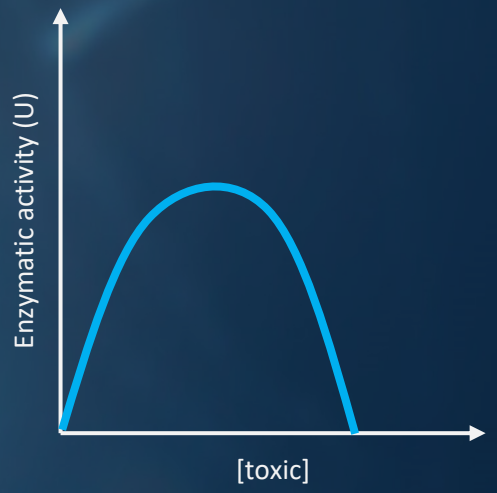
INVERSE PORPOTION

The activity of some enzymes can be impaired by certain toxic substances.



TYPICAL ENZYME KINETICS

The enzyme increases its activity in response to stress until a maximum velocity after which increasing toxic concentrations do not produce an effect.



PARABOLIC FUNCTION

The enzyme exhibits an increase in its activity until a certain concentration after which it is inhibited. The activity should be only used as biomarker in the positive slope range of concentrations.

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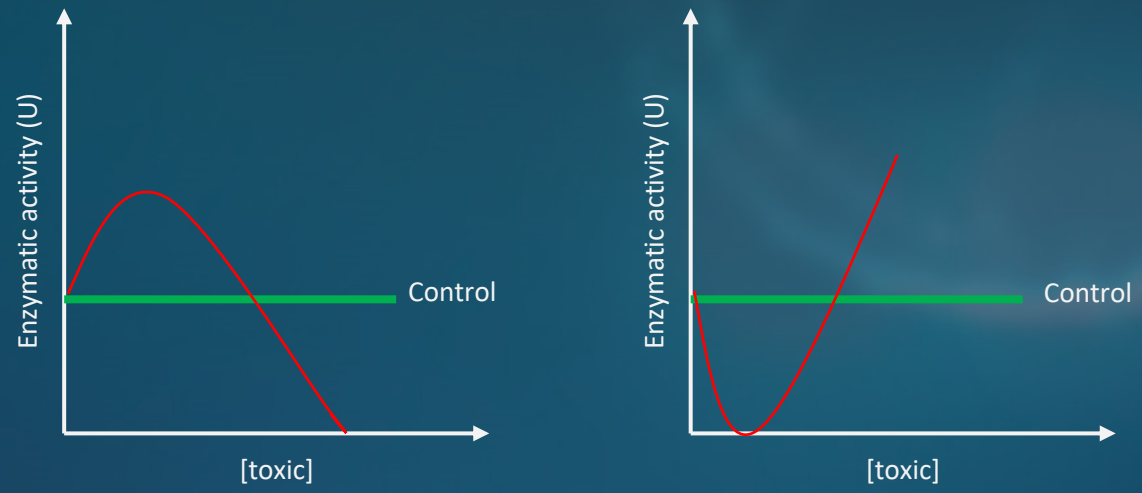

HORMESIS EFFECT

- Hormesis is any process in a cell or organism that exhibits a biphasic response to exposure to increasing amounts of a substance or condition.
- Within the hormetic zone, there is generally a favorable biological response to low exposures to toxins and other stressors.
- In toxicology, hormesis is a dose response phenomenon characterized by a low dose stimulation, high dose inhibition, resulting in either a J-shaped or an inverted U-shaped dose response.
- Such environmental factors that would seem to produce positive responses have also been termed "eustress".

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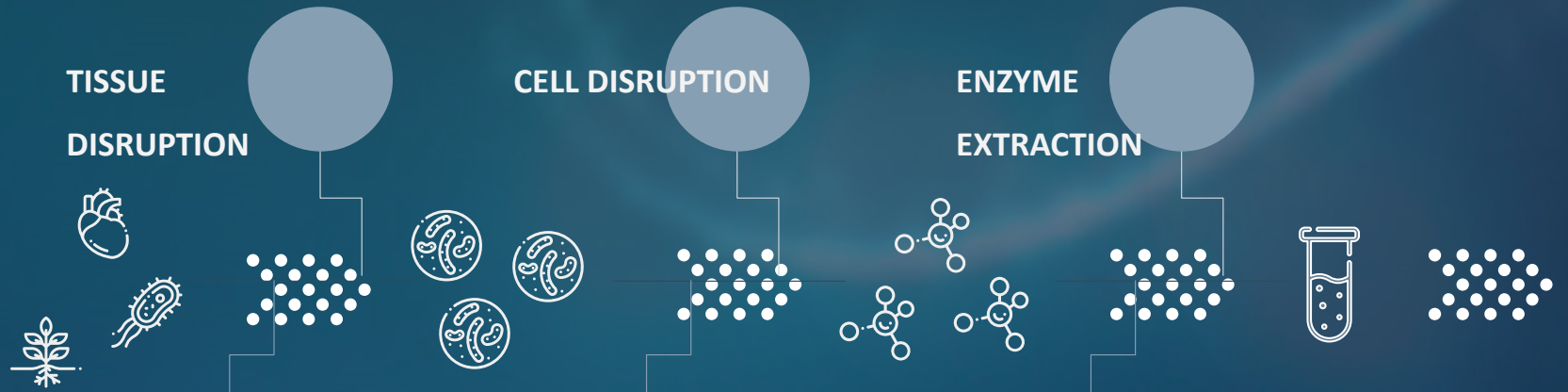
BIOMARKERS : ENZYME EXTRACTION



TISSUE DISRUPTION

N_2 or Quartz sand grinding, Tissue Lyzer

CELL DISRUPTION

Ultra-sound in physiological buffer with protection cocktail

(PBS typically supplemented with DTT, PVP and PMSF)

ENZYME EXTRACTION

Centrifugation and collection of the protein-containing supernatant

(Discard the pellet composed by cellular debris)

ENZYMATIC ASSAY

PROTEIN QUANTIFICATION

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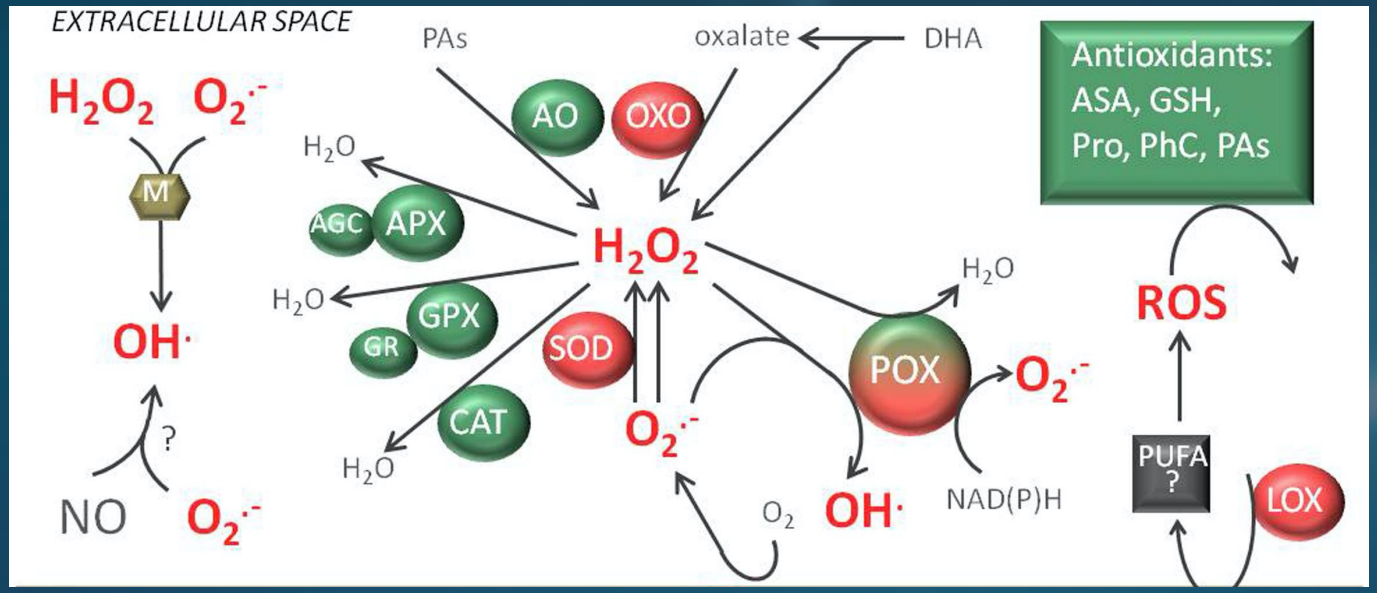
BIOMARKERS : OXIDATIVE sTRESS ENZYMES



- All these enzymes are part of the normal functioning of the cells.
- Cells produce ROS as part of their normal cellular metabolism.
- In Ecotoxicology, the activity of these enzymes towards the basal cell functioning is evaluated under stress conditions as a measure of the cell stress.

ASCORBATE PEROXIDASE (Apx, *ONLY PLANTS*)

Uses ascorbate molecules to quench the oxidative power from H_2O_2, converting hydrogen peroxide into H_20.

GLUTATHIONE PEROXIDASE (Gpx) AND REDUCTASE (GR)

Glutathione peroxidase used reduced glutathione molecules to converted H_2O_2 into H_2O. The oxidized form of glutathione (GSSH) is then reduced back by glutathione reductase at the expense of NADH.

CATALASE (CAT)

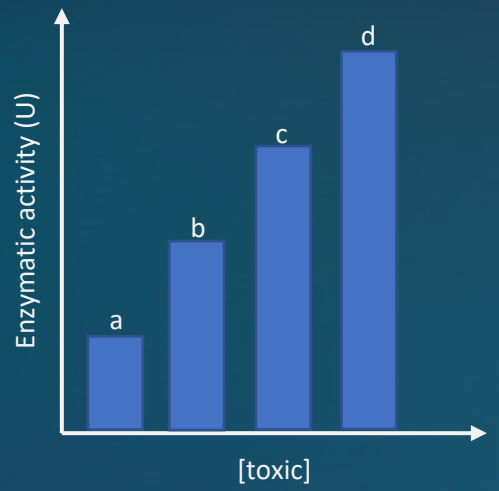
Converts hydrogen peroxide into water.

PEROXIDASE (POX)

A class of several peroxidasic enzymes that convert H_2O_2 into H_20 or OH^- at the expense of NAD(P)H.

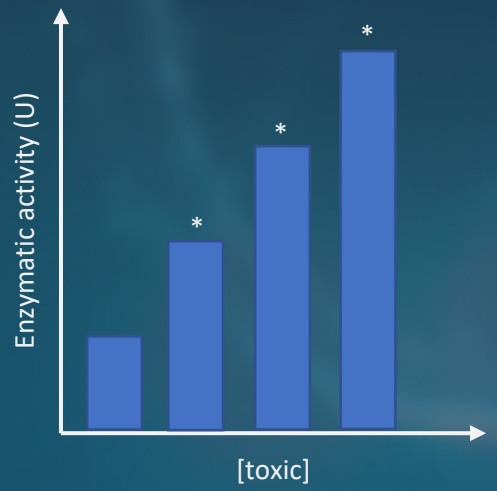
SUPEROXIDE DISMUTASE (SOD)

This enzymes converts the superoxide anion into hydrogen peroxide feeding the peroxidasic system..

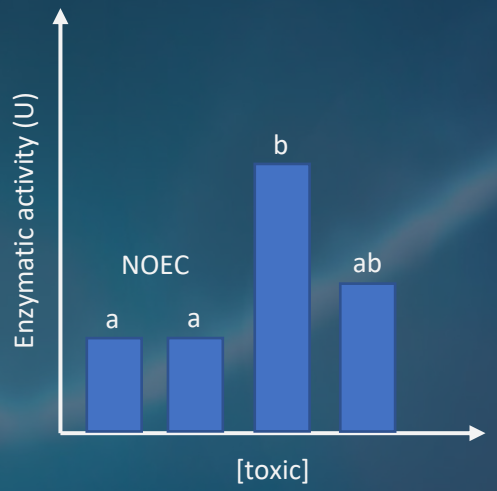


BETWEEN EFFECTS AND COMPARISON TOWARDS THE CONTROL

To evaluate incremental responses of the applied toxic towards all the tested concentrations (pair-wise comparisons).

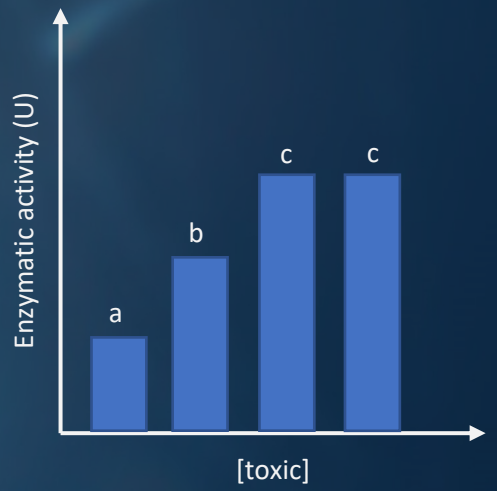


Classical ecotoxicology, comparison towards the control only.



NON-MONOTONIC

Linear increase until a toxicity threshold after which there is inhibition of activity or decrease of the biomarker concentration.



EXPONENTIAL

Linear increase until a toxicity threshold after there is a stabilization (saturation) and the biomarker stops responding.

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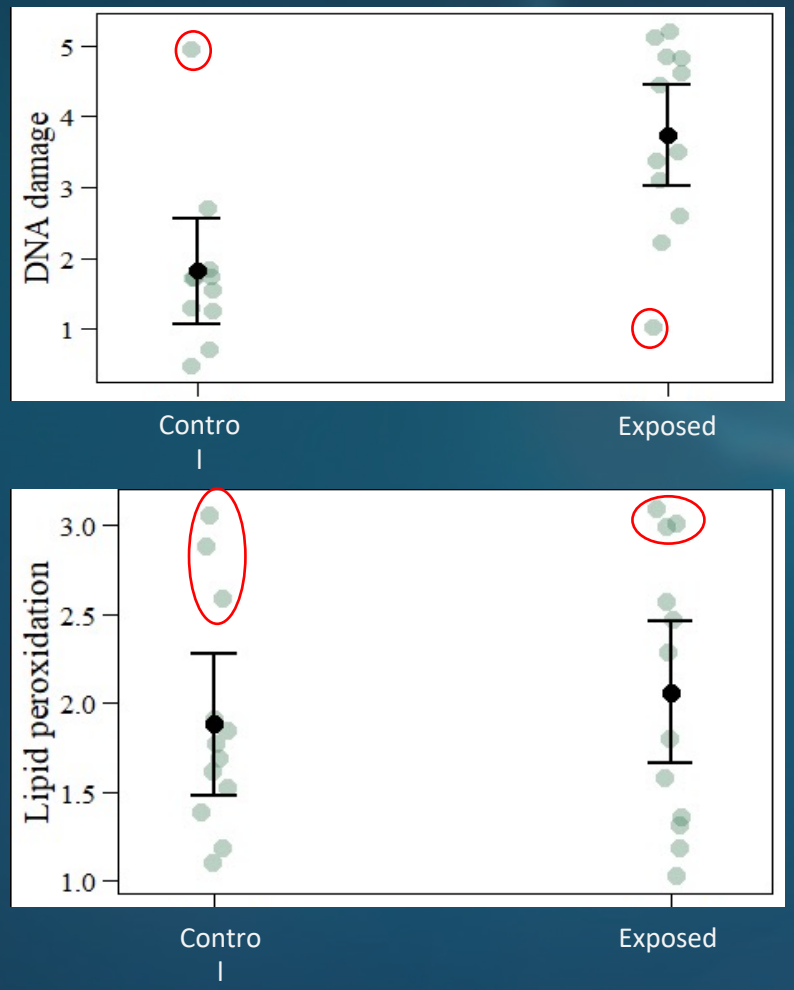

OUTLIER ASSESSMENT

- Non-clonal organisms have a high degree of variability.
- Operator performance and complex protocols can also introduce a degree of variance (besides biological replicates, technical analytical replicates are also recommended).
- It's important to evaluate the existence of severe or moderate outliers.

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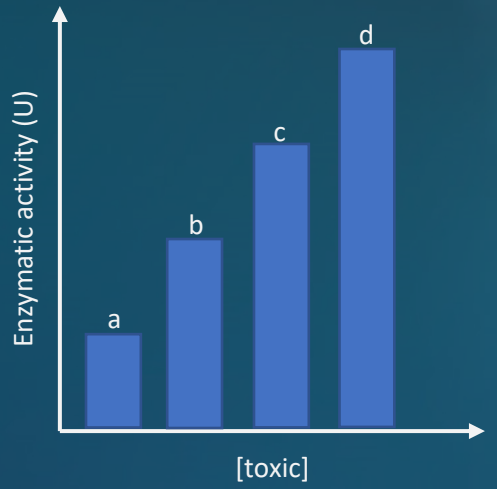
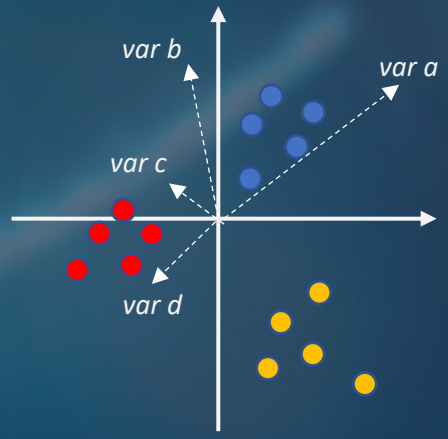
UNIVARIATE



Univariate is a term commonly used in statistics to describe a type of data which consists of observations on only a single characteristic or attribute. A simple example of univariate data would be the salaries of workers in industry. Like all the other data, univariate data can be visualized using graphs, images or other analysis tools after the data is measured, collected, reported, and analysed.

MULTIVARIATE



Multivariate statistics is a subdivision of statistics encompassing the simultaneous observation and analysis of more than one outcome variable. Multivariate statistics concerns understanding the different aims and background of each of the different forms of multivariate analysis, and how they relate to each other. The practical application of multivariate statistics to a particular problem may involve several types of univariate and multivariate analyses in order to understand the relationships between variables and their relevance to the problem being studied.

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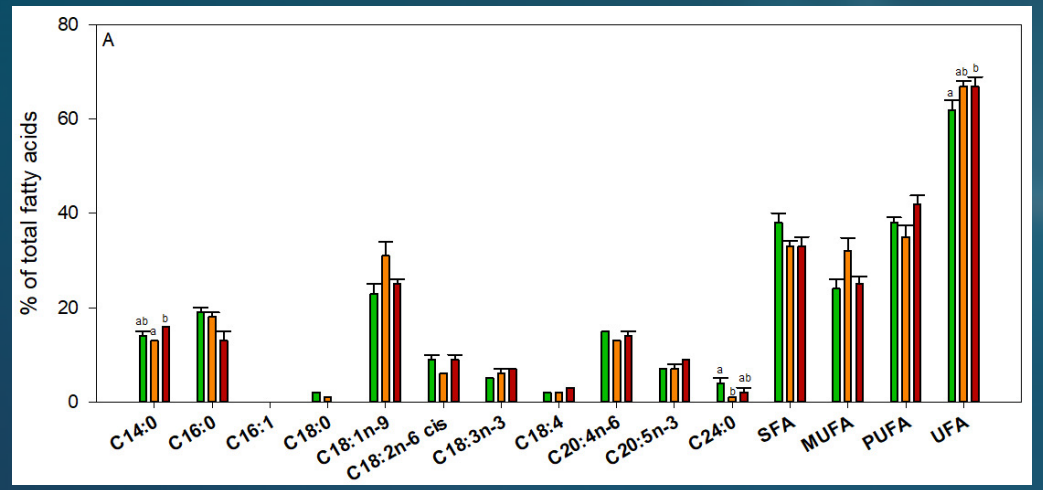
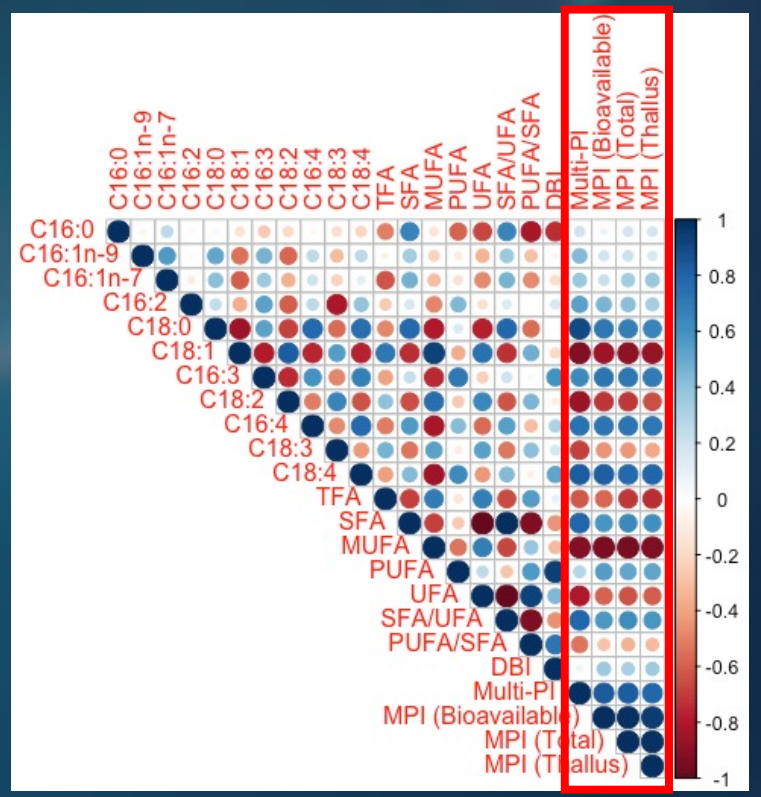
BIOMARKERS & STATISTICS: MULTIVARIATE STATISTICS

- **Multivariate analysis of variance (MANOVA)** extends the analysis of variance to cover cases where there is more than one dependent variable to be analysed simultaneously.
- **Multivariate regression** attempts to determine a formula that can describe how elements in a vector of variables respond simultaneously to changes in others.
- **Principal components analysis (PCA)** creates a new set of orthogonal variables that contain the same information as the original set. It rotates the axes of variation to give a new set of orthogonal axes, ordered so that they summarize decreasing proportions of the variation.
- **Factor analysis** is similar to PCA but allows the user to extract a specified number of synthetic variables, fewer than the original set, leaving the remaining unexplained variation as error.
- **Canonical correlation analysis** finds linear relationships among two sets of variables;
- **Redundancy analysis (RDA)** is similar to canonical correlation analysis but allows the user to derive a specified number of synthetic variables from one set of (independent) variables that explain as much variance as possible in another (independent) set.
- **Correspondence analysis (CA)**, finds a set of synthetic variables that summarise the original set, assuming chi-squared dissimilarities among records (cases).
- **Multidimensional scaling** comprises various algorithms to determine a set of synthetic variables that best represent the pairwise distances between records.
- **Discriminant analysis**, attempts to establish whether a set of variables can be used to distinguish between two or more groups of cases.
- **Linear discriminant analysis (LDA)** computes a linear predictor from two sets of normally distributed data to allow for classification of new observations.
- **Clustering systems** assign objects into groups (called clusters) so that objects (cases) from the same cluster are similar to each other.
- Recursive partitioning creates a **decision tree** that attempts to correctly classify members of the population based on a dichotomous dependent variable.
- **Artificial neural networks** extend regression and clustering methods to non-linear multivariate models.

NO APPARENT DIFFERENCES



CORRELATION ANALYSIS REVEAL SOME TENDENCIES



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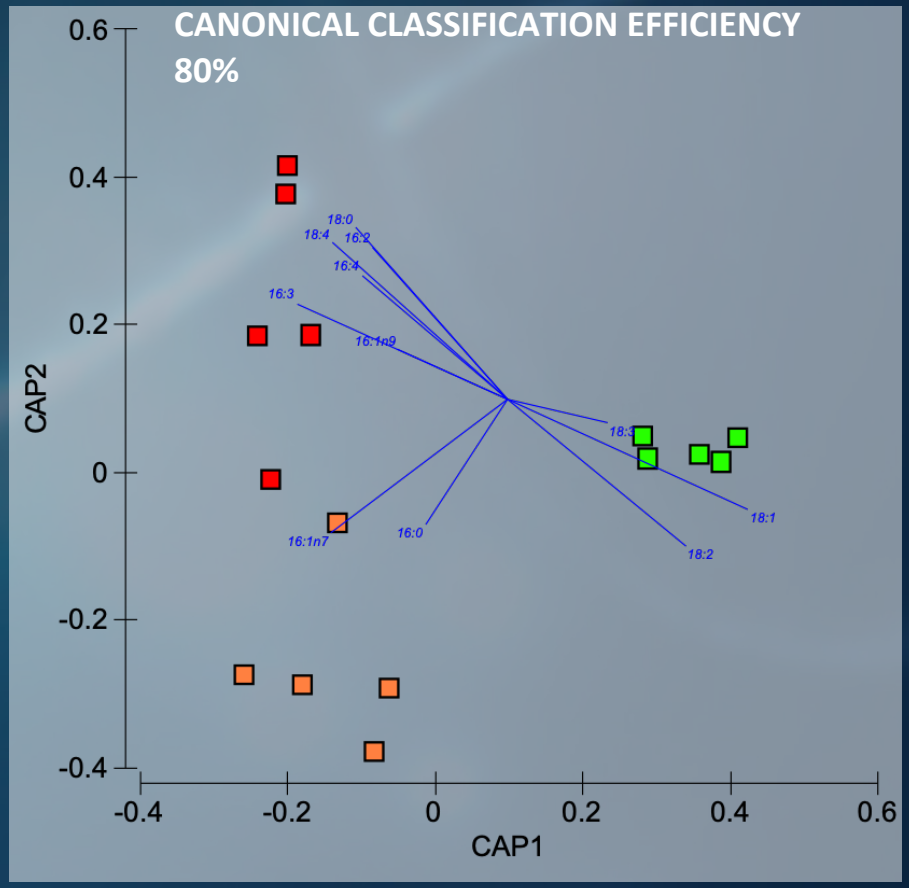
DISCLOSING NO APPARENT NO EFFECTS

- Called “CAP” for “Canonical Analysis of Principal coordinates,” this method will allow a constrained ordination to be done on the basis of any distance or dissimilarity measure.
- Canonical tests using permutations are also given, and we show how the method can be used
    - place a new observation into the canonical space using only interpoint dissimilarities,
    - to classify observations and obtain misclassification or residual errors, and
    - to correlate the original variables with patterns on canonical plots.
- Misclassification error or residual error is used to obtain a non-arbitrary decision concerning the appropriate dimensionality of the response data cloud (number of PCO axes) for the ensuing canonical analysis.



Orig. group	Class 1	Class 2	Class 3	Total	%correct
Class 1	4	1	0	5	80
Class 2	1	4	0	5	80
Class 3	0	1	4	5	80

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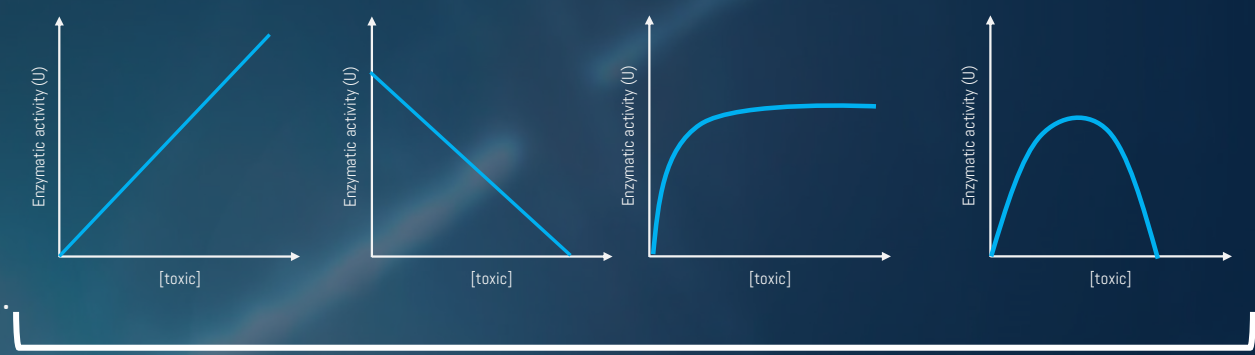
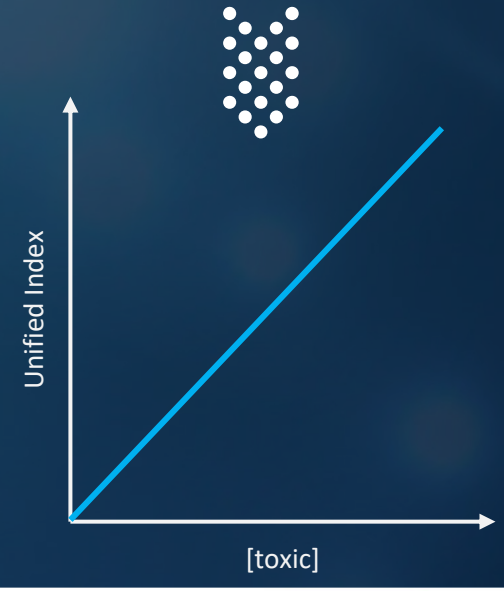
- Indexes aim to integrate a wide number of variables into a single numeric value.
- Advantages: easier communication to non-specialist audience of the ecotoxicological results attained, summarized into scaled values (for e.g. 1-0).
- Disadvantages: loss of physiological meaning.
- Variables must be normalized and weighted so that the mathematical value of a variable does not interfere just by having a different order of magnitude.
- Requires normally final index weighting using repetitive mathematical function (sin, cos for e.g.), power to an inverse number or logarithmic scales.
- Variable selection and weighting can be preformed having as basis a statistical approach or empirical knowledge.

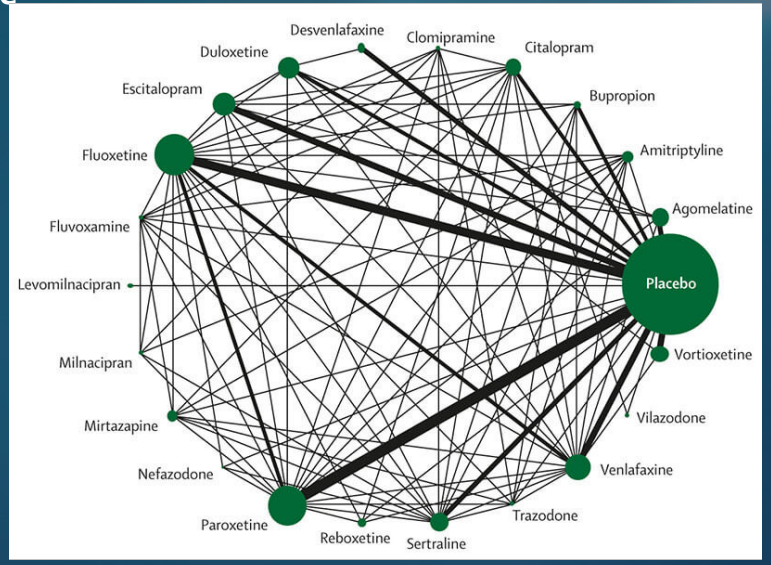


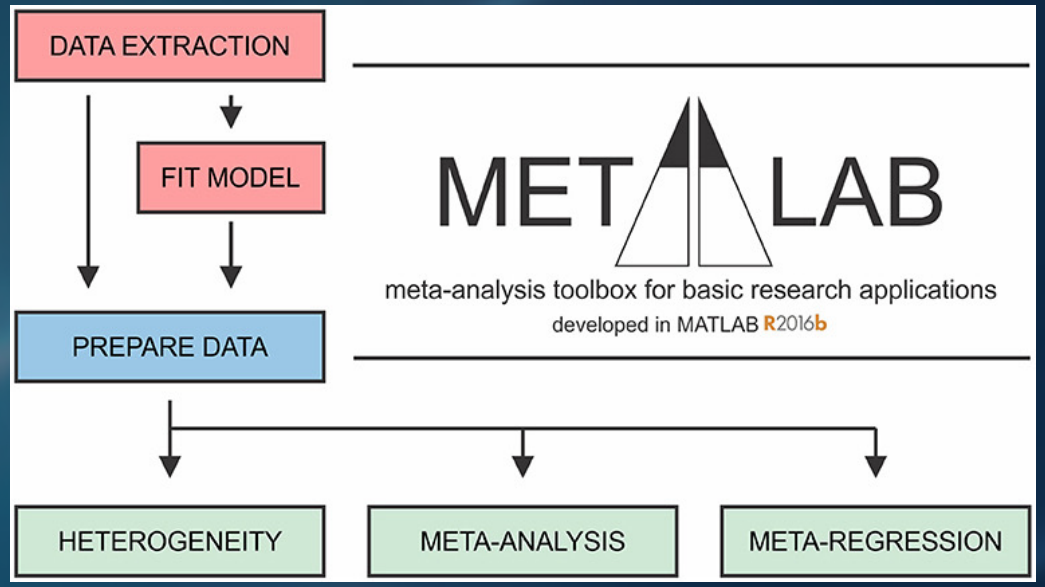
Set of individual biomarkers



Quantitative approach for systematically combining results of previous research to arrive at conclusions about the body of research.

- Quantitative : numbers
- Systematic : methodical
- Combining putting together
- previous research: what's already done
- conclusions: new knowledge





BIOMARKERS & STATISTICS: META-ANALYSIS

PUBLICATIONDATABASE ANALYSIS

Using scientific search engines (SCOPUS for e.g.) and appropriate keywords collected the highest number of references with data on the target subject.

PUBLICATION ANALYSIS

Analyse the scientific content of the studies and decide which ones to keep and to throw out according to a methodological decision tree.

METHODOLOGICAL EVALUATION

Evaluate the methods used and if they are preformed according to the predefined standards and adequate for being included in your comparative database.

DATA EXTRACTION

Extract all the data of the variables of interest: average, standard deviation, number of replicates.

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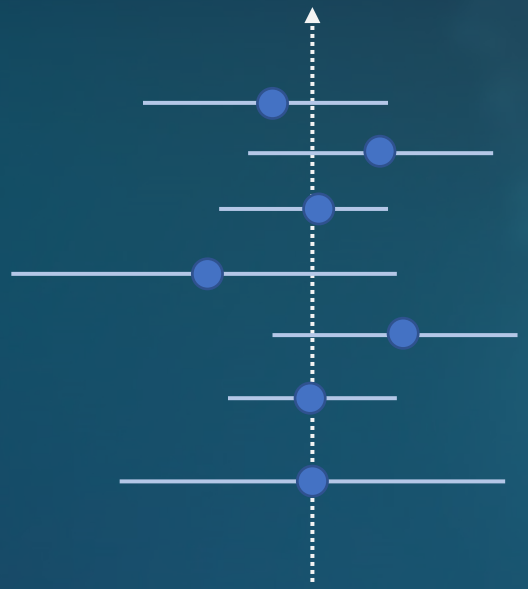
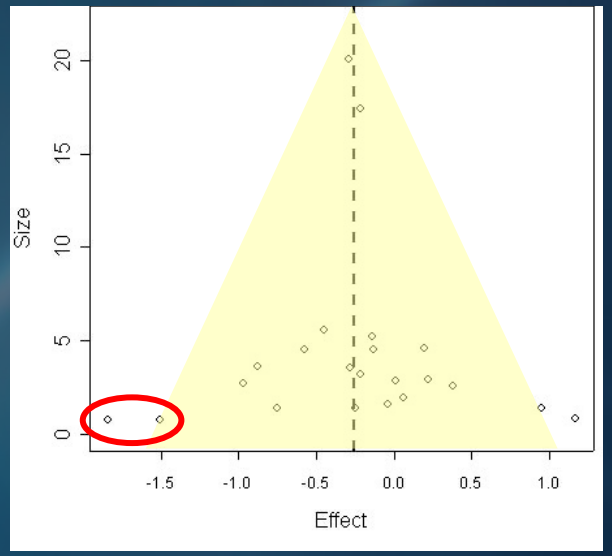
FOREST PLOT



- The dotted line passes across null, or 1.0
- The Risk Estimate of each study is lined up on each side of the dotted line, with 95% CI spread as the line

FUNNEL PLOT



- Plots the effect size against the sample size of the study
- To study a funnel plot, look at its LOWER LEFT corner, that's where negative or null studies are located
- If EMPTY, this indicates "PUBLICATION BIAS"
- Note that here, the plot fits in a funnel, and that the left corner is not all that empty, but we cannot rule out publication bias

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APPLICATIONS IN THE MARINE REALM

CASE STUDY EXAMPLES

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