PARTNER SEARCH PROFILE

1. Information about the call for proposals

Call for proposals and topic:

- Horizon 2020 Green Deal call: *Building a low-carbon, climate resilient future*
- Area 8: *Zero-pollution, toxic free environment*
- LC-GD-8-2-2020: *Fostering regulatory science to address combined exposures to industrial chemicals and pharmaceuticals: from science to evidence-based policies*

Deadline: January 2021

2. Proposal

Short description of the coordinator:

Aix-Marseille University (AMU) was created in 2012, resulting from the merger of the University of Provence, the University of the Mediterranean and Paul Cézanne University. It has more than 78,000 students including 10,000 international students, 7,680 faculty and staff members, 12 doctoral schools and nearly 3,300 PhD students. AMU is the coordinator of the Erasmus + European University Alliance "CIVIS". AMU has been involved in more than 100 FP7 projects and until now 102 H2020 projects.

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Title: *Improvement of experimental models using invertebrate animals to assess the environmental toxicity of very low doses of chemicals mixtures*

Project description including if possible Work Packages organisation

Introduction

Our planet is now in the midst of its sixth mass extinction of plants and animals and unlike the previous five, this one is of anthropogenic origin. Some scientists argue that extinction is normal,

simply an inevitable consequence of the process of evolution. However, on one hand the rate is 100 times that of the "normal rate" throughout geological time, and in the other hand the current extinction is not an inevitable consequence of the process of evolution, it is the consequence of a too rapid change in the environment that has not allowed many species to adapt to it [1-2].
Epidemiological data show in developed country over the past three decades an increased incidence of allergic and auto-immune disease (ex: type I and type 2 diabetes pandemies), and of some cancers (ex: in 20 years the incidence of breast cancer has doubled) [3].
We are therefore witnessing the emergence of both the notion of extinction and diseases of Anthropocene. Environmental pollution by chemicals, including Persistent Organic Pollutants (POPs), pesticides, endocrine disrupters..., play a major role in these processes. Moreover, it underscores the interest of global ecology, where we do not separate the health of our planet from that of humans. Therefore, a multidisciplinary approach is necessary to study and manage globally the consequences of environmental pollution.
Generally, ecotoxicological studies performed in animals are limited to the impact of a unique pollutant on the behavior, the morphology and the reproductive rate of the studied species. Cocktail effects are poorly studied, as well as the biochemical, transcriptomic and epigenetic consequences. In the environment, living beings, including humans, are exposed to numerous chemicals compounds and studying the effect of only one pollutant is not relevant. Major signaling pathway are conserved among evolution [5-7] (ex: Wnt pathway, insulin signaling, TOR signaling…) and depicting molecular mechanisms in lower animals would be of interest to predict some detrimental effects in humans. In a different field, there is also a desire at the European level to ban the animal experiments performed in mammals to assess the toxicity of chemical compounds, in favor of alternative methods. A better knowledge of biochemical and molecular mechanisms will be of great help in their development.
The aim of our project is to study the detrimental effect of environmental pollutants (alone or in combination), by means of two animal models largely used in ecotoxicology, an aquatic one *Hydra vulgaris* (hydrozoan cnidarian) and a terrestrial one *Cantareus aspersus* (pulmonate mollusk). We will study two POPs, exhibiting endocrine disrupter (ED) properties, that deeply impact environmental and human healths: chlordecone (CLD) and PCB118, as well as two decried systemic pesticides glyphosate and boscalid. The effects will be characterized by a classical approach (behavior, morphological changes, reproduction…) and by an original one involving coupled biochemical, transcriptomic and epigenetic approaches.

<u>Presentation and rational of models used</u>

The prerequisites for the choice of our models were to choose a species that is sensible to pollutants, easy to breed, where the exposures would produce little waste that should be eliminated, whose genome has been sequenced, and lives in aquatic or terrestrial environments.
Hydra sp. has been widely studied due to its regenerative properties, but also as a model in ecotoxicological studies. The table below summarizes the advantage of this model:

Hydra sp. characterisitics	Advantages
Diploblastic	All the cells are in contact with the medium High sensitivity towards xenobiotics
Small size	Low sized rearing zones Low xenobiotics volumes Little amount of wastes to dispose
Clonal reproduction	Rapid formation of large colonies Smoothing of inter-individual variability
Invertebrate	Qualification not mandatory for technicians Ethic committee validation not mandatory

We have already used *Hydra circumcicta*, in a previous study [8] to evaluate the impact of environmental concentrations of chlordecone on the expression of some stress genes and have observed non monotonous dose response curves in agreement with its endocrine disruptive properties (Fig.1A). Moreover, in preliminary work we have observed that the main small-non-coding RNA regulation pathway not to be the miRNA pathway but seems to be the PIWI-interacting-RNA (piRNA) pathway (Fig.1B). The small RNA sequencing data are still under analyzes.

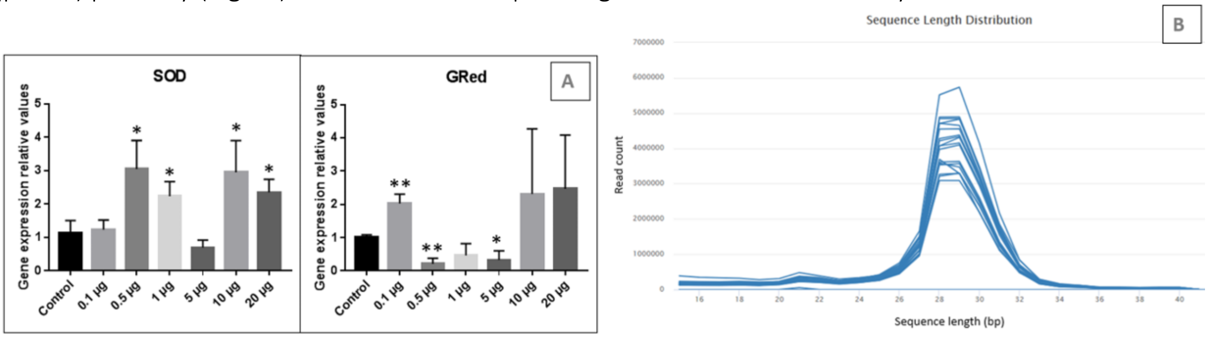

Fig. 1: A: effect of environmental concentration of chlordecone on the relative gene expression of superoxide dismutase (SOD) and glutathione reductase (GRed) after 14 days of exposition [8]. B: Number of read counts by sequence length for all the samples analyzed. Each line corresponds to a unique sample (Colpaert et al., unpublished data).

The land snail *Cantareus aspersus* is a well-known good indicator of terrestrial environment quality and of the chemical transfer risk in the ecosystems [9]. The laboratory of “Chrono-environnement” (LCE UMR6249) has a strong experience of this model and its use in *in situ* and *ex situ* ecotoxicological assessment of chemicals and soil contamination [10]. The table below summarizes the advantage of this model:

Cantareus aspersus characterisitics	Advantages
Relatively rapid reproduction rate	High number of individuals for the experiment
Small sized	Reasonable space dedicated to rearing In-situ and ex-situ tests easy to set
Integration of three contamination pathways	Opportunity to assess the contamination Pathways one by one or simultaneously High accumulation capacities
Invertebrate	Qualification not mandatory for technicians Ethic committee validation not mandatory

Among the different studies carried out during the last decade, the laboratory of “Chrono-environnement” has, for instance, demonstrated, based on sub-lethal individual endpoints (growth, sexual maturation, fecundity and fertility), that glyphosate exhibits ED properties in this species, especially when snails were exposed during early life stages (Fig. 2). From a molecular point of view, they also already explored the relevance of coupling Random Amplified Polymorphic DNA (RAPD) and a High-Resolution capillary electrophoresis System (HRS) method for assessing the genotoxic potential of a wide variety commercial formulations of pesticides [11-12]. However, regulation pathways still need to be characterized.

The sea urchin, *Echinometra lucunter*, is widely used in embryo-larval tests for ecotoxicological studies, are generally studied in this model fertilization and embryogenesis [13-14]. The table below summarizes the advantage of this model:

Sea ursins characterisitics	Advantages
Relatively rapid reproduction rate	High number of individuals for the experiment
Small sized	Low sized rearing zones Low xenobiotics volumes Little amount of wastes to dispose
Widely used and validated model to assess marine pollutants toxicity	High accumulation capacities Transparency of embryos that allow the direct observation of cell division and movement inside the living embryos and larvae Sea urchins are more closely related to humans than other invertebrates
Invertebrate	Qualification not mandatory for technicians Ethic committee validation not mandatory

Choice of the studied pollutants

We have chosen in this study, 3 widely used POPs exhibiting ED properties: an organochlorine insecticide, CLD, and two industrial compounds, PCB118 (dioxin-like compounds) and perfluoro-octane acid (Per- and polyfluoroalkyl substance).
CLD was used from 1973 in French West Indies to control the banana root borer *Cosmopolites sordidus,* until its interdiction in 1993. The bishomocubane structure of CLD limits its degradation in the environment. CLD is strongly sorbed to organic matter, and soils and water are contaminated for decades to centuries. In humans and animals, the exposition mainly occurs through the consumption of contaminated food and water. Epidemiological studies have associated CLD exposure to a shortened gestational length and increased risk of preterm birth, children cognitive and motor developmental issues, as well as prostate cancer [15]. These ED effects were probably mainly related to its estrogenic property.
Polychlorobiphenyls (PCBs) were first commercially produced in the United States in 1929 and used widely in capacitors, transformers, hydraulic fluids, heat transfer fluids, lubricants, plasticizers and as components of surface coatings and ink. An international ban on production was enacted at the Stockholm Convention on POPs in 2001. PCBs undergoes a slow degradation in the environment. Their persistence increases with chlorination degree, and they are mainly trapped in organic matter, notably in water. Rivers were contaminated mainly by industrial discharges. PCBs are divided into dioxin-like PCBs (PCB-DL) and bulky PCBs. Both exhibit ED properties, but PCBs-DL appear the most detrimental. Their exposure is associated to an increased risk of numerous pathologies in humans, including cancers, hypothyroidism, cognitive and behavioral impairments, type 2 diabetes. PCBs-DL toxicity was probably linked to the activation of Ah receptor [16]. 7 “indicator PCBs” were defined at European level, representing 80% of environmental PCBs, among them PCB118 is the sole PCB-DL.
Per- and polyfluoroalkyl substances (PFASs) are synthetic chemical compounds with multiple fluorine atoms attached to an alkyl chain. Introduce in 1940’s, they have been widely used as fluorosurfactants to produce fluoropolymers. Epidemiological studies have associated exposition to PFAS with various diseases: hypercholesterolemia, cancer, ulcerative colitis, thyroid diseases, pregnancy-induced hypertension and pre-eclampsia. Their persistence, toxicity, and widespread occurrence in the blood of general populations and wildlife have focus the attention of regulatory agencies [16-17]. They were classified as POPs in 2009.
The two other studied pollutants are not POPs but are two decried pesticides: glyphosate (herbicide) and boscalid (fungicide). Glyphosate was strongly suspected to be cancerogenic, boscalid is the most SDHI fungicide used. SDHI fungicides inhibits the grow of fungus by inhibiting the respiratory chain. This latter property, associated to a poor knowledge of the effect on health and environment, has led

to the fear of these pesticides. Glyphosate and boscalid are not persistent in our environment, but are present in both water and soils, their systemic properties allow their absorption by plants that are further consumed by animals, including humans.

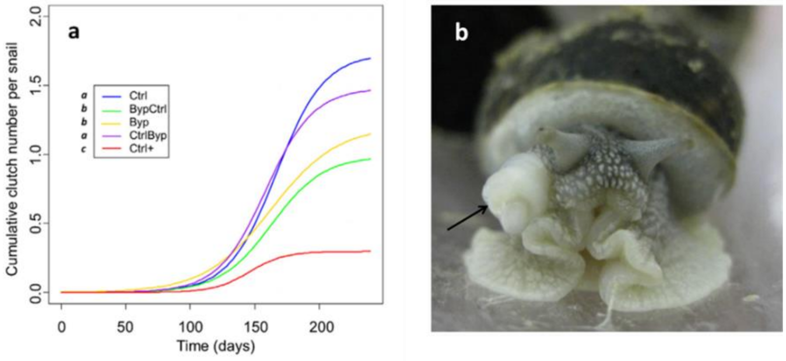

Fig.2: a: Logistic modeling of the time course of the cumulative clutch number per snail for the different treatments (Bypass® applied during embryogenesis and/or during rest of the life cycle). Different letters indicate significant differences between treatments ($p < 0.05$). b: Exposed snail with the genital tract externalized from its body (indicated by an arrow). From [11].

<u>Experimental procedure</u>

<u>Pollutant expositions</u>

Concentration used of studied pollutants

Hydras, snails and sea ursins will be firstly exposed to a single pollutant at environmental concentrations. We will study three concentrations to assess the concentration-effect relationships. However, living animals are exposed through the environment to numerous pollutants and therefore it is of interest to evaluate cocktail effects. To limit the number of experiments to set the concentration of each pollutants, we will use plan of experiments. An experimental design is a reasoned organization of tests that allows to guarantee the quality of the information coming from the experiments, while considering the technical and economic constraints of the study [19]. That will allow us to organize our experiments, considering the quantitative nature of the factors which induces a number of infinite combinations in the range of variation associated with them. For this, we will vary two factors simultaneously and quantify a possible interaction effect between them. Finally, we will develop a predictive mathematical model whose coefficients will be estimated with the smallest possible variance, while reducing the number of experiments to a minimum.

Hydra exposure modalities

We will use two clones of *Hydra vulgaris*, a male and a female one, bred under controlled condition at IMBE. Hydras will be exposed during about two weaks, in order to be able to measure the biological parameters, especially the reproductive ones. At the end of the exposition, medium and a sample of Hydras will be collected to quantify the concentration of pollutants.

Snail exposure modalities

Snails will be bred under controlled condition at Chrono-environnement. Various developmental stages will be used in life cycle experiments: embryos, juveniles and adults. The exposure source will be the soil through two main routes: cutaneous (diffusion through the egg wall or the skin of the foot) and digestive (ingestion of soil particles). At the end of the exposition, medium and samples of snail hepatopancreas will be collected to quantify the concentration of pollutants.

Sea ursin exposure modalities

We are looking for a partner for Sea ursins approaches. Generally, are used isolated gametes, and larvae.

The studied pollutants will be quantified in the in the different matrices to evaluate their biodisponibility in each studied model (LC-MS-MS).

Evaluation of morphological and/or reproductive rate

Concerning hydras, we will study the predatory behavior, the induced morphological modifications (depending on the toxicity, six stage were described [20]), and the modification of asexual reproductive rate.

Concerning snails, different biological endpoints, at various levels of biological organization, will be investigated:

- embryotoxicity, using several morphological and physiological endpoints in embryos exposed and monitored during about two weeks: size, heart rate, delay in hatching, states of development of non-hatched eggs and the fresh mass of newly hatched embryos [21]
- growth, according to the standardized ecotoxicity bioassay [22]. During four weeks of exposure, individual fresh mass and shell diameter of juvenile snails will be monitored. Impacts will be assessed using modeling approaches allowing to describe growth inhibition according to the maximal mass reached, the growth constant and the time at the inflection point.
- reproduction, using several complementary endpoints monitored on sub-adult and adult snails [23]: sexual maturation (according to the acquisition of a lipped shell, corresponding to the end of the growth period and the maturation of sexual organs), fecundity (*i.e.*, total number of eggs laid per snail) and fertility (*i.e.*, the total number of eggs hatched per snail). As for growth, models will be fitted to the data to check for developmental and reproductive impairments.

Concerning sea ursins, we are looking for a partner. Generally; toxicity tests are performed on isolated gametes that are firstly examined under the microscope and the ability of fertilization was further confirmed by egg insemination. The fertilization test is a short, yet sensitive test with sea urchin spermatozoa, and it would be performed according to the USEPA 2002 guideline. Sea urchins generate rapidly developing, morphologically simple, and optically transparent larvae and are a well-established model system supported by a broad array of genomic resources, experimental approaches, and imaging techniques. As such, they provide a unique opportunity to study postembryonic processes such as endocrine signaling, immunity, host-microbe interactions, and regeneration.

Evaluation of biochemical parameters

Biochemical parameters will be estimated in order to complete the data obtained by the transcriptomic and epigenetic approaches. Firstly, we will assess the induced oxidative stress (ex: catalase, superoxide dismutase, glutathione peroxidase activities; malone dialdehyde production), the EROD activity (catalyzed by CYP1 that bioactivates procarcinogens), the LDHI activity, the acetylcholinesterase activity, the genotoxicity. Other relevant biochemical parameters will be defined after the analysis of transcriptomic and epigenetic data.

Evaluation of the effects of exposition on the gene transciption and little RNAs expression

At the end of the expositions, the effect of expositions will be evaluated at a transcriptomic (mRNAs) and epigenetic (piRNAs and miRNAs) by high-speed sequencing. Little RNAs are currently considered as one of the major elements in the control of genes encoding within the genome. By targeting and controlling mRNA expression, miRNAs can highly sophisticated modulates intracellular signaling pathways as well as other biological pathways. Simultaneous study of mRNAs and miRNAs in the same sample is now possible with the NEXTFLEX Combo-Seq Kit that generates combined mRNA and small RNA libraries in a single workflow total RNA inputs. We propose that the work will be done at the TGML platform that is a founding member of the National Infrastructure for genomic analysis and bioinformatics France Génomique. In order to set up the combo-seq analysis pipeline and analyze the data, a bioinformatician is essential. There is a need of scripting skills to write a reproducible and robust pipeline and also a need of statistical skills to enable the data analysis (i.e differential expression analysis, hierarchical clustering, …).

This will allow to highlight main molecular targets of studied pollutants, used alone or as cocktails. Phylogenetic studies will be further undertook to evaluate putative targets in mammals, including humans.

Workpackages

WP1: Hydra expositions (IMBE UMR7263), evaluation of morphological and reproductive rate, relevant biochemical parameters, plan of experiments

WP2: Snail exposition (LCE UMR6249), evaluation of morphological and reproductive rate, relevant biochemical parameters

WP3: Sea ursin exposition (looking for partner), evaluation of morphological and reproductive rate, relevant biochemical parameters

WP4: Transcriptomic and little RNAs expression (TGML, TAGC U1090)

WP5: Quantification of studied pollutants by LC-MS-MS in the in the different matrices (looking for partner)

WP6: Phylogenetic studies (looking for partner)

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3. Profile and expected role of the partners sought:

We are looking for partners having the following profile:

- Academic/Non-academic
- Ecotoxicological laboratory specialized in sea ursin model (WP3)
- Analytical laboratory specialized in LC-MS-MS quantification in different matrices (WP5)
- Laboratory specialized in phylogenetic analyses (WP6)

Preferably coming from following countries or regions: Europe, USA, Canada, Japan

The selection of the partner will take into account:

- The scientific competence
- The added value to the project