PROPOSITION DE SUJET DE THESE

1. Titre du projet

Acronyme du projet : BB

Approche respectueuse de l'environnement pour la **B**iorestauration des sols contaminés et la production de **B**iostimulant : Contribution potentielle des algues marines côtières européennes à l'amélioration de l'environnement.

An ecofriendly approach for **B**ioremediation of contaminated soil environment and production of **B**iostimulant: Potential contribution of European coastal seaweed to environmental improvement.

2. Présentation de l'établissement porteur

Établissement porteur du projet : Université Bretagne Sud

Ecole Doctorale : Sciences de la Mer et du Littoral

3. Encadrement

Nom du laboratoire d'accueil : Laboratoire de Biotechnologie et Chimie Marines

Code du laboratoire (U/UMR/USR/EA/JE/…) : EA

Directrice du Laboratoire : Nathalie Bourgougnon

Nombre HDR dans le laboratoire : 8

Nombre de thèses en cours : 10

Nombre de post-docs en cours : 3

Website : http://www-lbcm.univ-ubs.fr/fr/index.html

The Laboratory of Marine Biotechnology and Chemistry Laboratory is a laboratory of the Université de Bretagne-Sud (UBS) (University of South Brittany) which is related to the Higher Education and Research Unit in Sciences and Engineering Sciences. Although it celebrated its 20th anniversary in 2015, UBS is one of the most recent French universities, created by merging technical schools in Vannes, Pontivy and Lorient. LBCM was awarded the Equipe d'Accueil label in 2000 and given an A grade in 2006, 2011. The LBCM is integrated into the IUEM. LBCM has been heavily involved in higher education since its creation and has developed different undergraduate and postgraduate degree courses over the years in particular the postgraduate degree, the general three-year degree courses (Licences) "Biotechnology" and "Chemical and Biological Analysis Techniques", a professionally oriented degree course (Licence pro) "Development of Cosmetic Products and Health", and for the last 11 years a Master's degree related to biotechnologies), and has also trained young researchers (>30 PhD theses defended since 2010), students (many interns at undergraduate and postgraduate levels). The LBCM is organized into two research axes (1) biofilms and marine (2) blue biotechnologies combining biological and chemical approaches. The LBCM has a pool bioassays and tools for the design, characterization, formulation and development of innovative technologies in the areas of antibiofilm paints, cosmetic dosage forms, and health. LBCM also holds the specific relevant equipments (confocal laser scanning microscope, mass spectrometers LC-MS, MALDI-TOF, microbiological – virological safety stations, centrifuge machines; HPLC, CPG, spectrophotometers). The group has also patented several molecules and was involved in national and international programs. LBCM is involved in Tremplin Carnot Agrifood transition.

Nom et prénom du directeur de thèse (HDR), porteur du projet : Professeure Nathalie Bourgougnon

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Nathalie Bourgougnon has a recognized know-how in marine biomolecules extracted from seaweeds and biofilm. It possesses a good knowledge on French invasive algae (particularly on physiochemistry of *Solieria* species), extraction and purification of marine substances, screening of biological activity and valuation opportunities (cosmetics and healthcare fields). She has published >90 in peer-reviewed journals, 10 chapters, 2 patents about antiviral substances (https://scholar.google.fr/citations?user=0_LDGF4AAAAJ&hl=fr).She has been involved in several European projects (e.g. FP4 Bioactive Marine Natural Products in the Field of Antitumoral, Antiviral and Immunomodulant Activity, MAST III; FP5 Anti Viral Infection Non Specific Immunity; FP6 Biotechnology valorization of the marine resources; FP7 Biotechnological exploitation of marine products and by-products and BIOVADIA PEOPLE-2010-IRSES) and recently in OSEO, different FUI program and international programs (ECOS Nord, PHC Indonesia). She has received the national research and PhD supervision award (PES/PEDR) since 1999.

Recent Publications:

Kevin Hardouin, Gilles Bedoux, Anne-Sophie Burlot, Pi Nyvall-Collen, **Nathalie Bourgougnon** (2014). Enzymatic recovery of metabolites from seaweeds: potential applications. *Advance Botanical research,* Volume 71, Pages 279-320

Kulshreshtha G., Burlot A.S., Marty C., Critchley A., Hafting J., Bedoux G., **Bourgougnon N**., Prithiviraj B. (2015). Enzyme-assisted extraction of bioactive material from *Chondrus crispus* and *Codium fragile* and its effect on *herpes simplex* virus (HSV-1). Marine Drugs 13, 558-580

Kevin Hardouin Gilles Bedoux, Anne-Sophie Burlot, Claire Donnay-Moreno, Jean-Pascal Bergé, Pi Nyvall-Collén, **Nathalie Bourgougnon** (2016). Enzyme-assisted extraction (EAE) for the production of antiviral and antioxidant extracts from the green seaweed *Ulva armoricana* (Ulvales, Ulvophyceae). Algal Research 16 (2016) 233–239

Burlot Anne-Sophie, Bedoux Gilles and **Bourgougnon Nathalie** (2016). Response Surface Methodology for Enzyme-Assisted Extraction of Water-Soluble Antiviral Compounds from the Proliferative Macroalga *Solieria chordalis*. Enzyme Engineering. 5:2

Ana Peñuela, Daniel Robledo, **Nathalie Bourgougnon**, Gilles Bedoux, Emanuel Hernandez, Yolanda Freile-Pelegrín (2018). Environmentally Friendly Valorization of *Solieria filiformis* Gigartinales, Rhodophyta) from IMTA using a Biorefinery Concept. Marine drugs *in press*

-Co-directeur-trice de thèse et co-encadrant scientifique : Izabela Michalak (Assistant Professor at WUST from 12 December 2018)

- Laboratoire de recherche co-encadrant (nom + code U/UMR/USR/EA/JE/...) Wroclaw University of Science and Technology – WUST, Faculty of Chemistry, Department of Advanced Material Technologies, Wroclaw, Poland,

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Izabela Michalak (https://scholar.google.fr/citations?user=ONsds1AAAAAJ&hl=fr) from Wroclaw University of Science and Technology (Poland) has good skills in the testing of utilitarian properties of algal extracts in germination test, pot experiments, as well as in field trials. She has also experience in the utilization of the algal biomass as a biosorbent of metal ions. By combining our respective skills, we would like to carry out a laboratory scale preparation of algae extracts from Poland and French biomass (France) and an analysis of biostimulant properties of algal extracts and biosorption properties of the examined biomass (Poland). Notably, she is known for two famous publications:

Michalak, I., & Chojnacka, K. (2015). Algae as production systems of bioactive compounds. *Engineering in Life Sciences*, 15(2), 160-176.

Michalak, I., Tuhy, Ł., & Chojnacka, K. (2015). Seaweed extract by microwave assisted extraction as plant growth biostimulant. *Open Chemistry*, 13(1).

4. Project summary

Over the last 20 years, agricultural strategies all around the world have been adapted to the changing environmental conditions by modifying and diversifying its production system by using chemical fertilizers, pesticides, and water management. However, recent data of soil qualities and forecasted agro-hydrological change threaten the availability of these farming and social systems and subsequently food security in lot of countries. There are significant constraints that limit the ability of the farmer to adapt the suitable cultivars to certain specific conditions. These constraints include soil nutrient management, lack of knowledge of potential threats from acid or saline soil, emission of greenhouse gases from different types of culture field in aerobic or anaerobic condition etc.. Improvements made in agriculture usually lead to a larger production capacity which can therefore increase the environmental impact. For example, pollution due to toxic metals and its implications for public health and the environment have led to the increased interest in the developing of environmental biotechnology approaches. In a food challenge context of increased competition on raw materials, environmental challenges and public health shared globally, the **strategic character of agriculture**, also carries geopolitical dimensions. The agriculture resonates worldwide with growing food demand and increased competition in emerging countries. The research, development and innovation are the main drivers of competitiveness of companies in this sector in Europe.

Currently, there is a **tendency to search for a new generation of agro-products manufactured on the basis of raw materials of biological origin**. Among the marine flora encountered in Europe, some macroalgae can become invasive or proliferative and have profound adverse ecological impacts. Due to unique composition of algae, this underexploited biomass can be used to manufacture agricultural products that are safe for consumers and natural environments (no ecotoxicological, toxicological hazard, residues on agricultural products). **Algal products (e.g., extracts)** that improve the uptake of minerals from the soil and increase the resistance of plants to biotic and abiotic stress can contribute to the **reduction of chemicals used in agriculture**. Combined with more precise dosing of mineral fertilizers and synthetic plant protection products, they will have a very positive impact on the natural environment.

Thus, **the development of innovative products and processes, but also the valorization of by-products,** may allow them to differentiate by improving their image, the quality and their margins. However, the management of environmental and health issues is a key element for competitiveness and sustainability. To this respect it is important to introduce the **biorefinery** concept that integrates biomass conversion processes in value-added chemicals from biomass. Nowadays, the concept of biorefinery involves the development of eco-friendly processes for sequential recovery of molecules, especially bioactive molecules and **while preserving most of the intrinsic qualities of raw material**. We propose to choice a Green Chemistry approach combining selectivity, cost-effectiveness, and an eco-friendly process. To maximize the added value of the biomass by allowing the co-extraction of valuable components, a strategy of biorefinery will be developed at lab and industrial levels.

The aim of the project is to develop an innovative biorefinery concept with invasive European seaweeds to produce a sustainable liquid biostimulant of plant growth that will improve crop yield and quality. Unexploited algal biomass collected from the seashore, as well as post-extraction residues from the isolation of biologically active compounds from seaweeds can be also used in the bioremediation of polluted water (with the use of biosorption process) and soil.

5. Context Objectives and scientific interests

5.1 Hypothesis and Bottlenecks

Hypothesis 1. Seaweed extracts will act as biostimulants of plant growth: Plant growth and development relies on the availability of a favorable growing environment, which includes healthy soils, availability of nutrients, aswell as protection against pests and other stresses. These conditions can be met naturally or provided artificially. The main problem is an inappropriate soil quality which therefore leads to limited food production. Amongst various methods used to improve crop growth and development, the use of plant biostimulants has become more and more significant in the recent years. Biostimulants are materials, other than fertilizers or plant nutrients, which are able to stimulate plant growth and development when applied in small quantities. They are also referred to as 'metabolic enhancers'. Amongst a wide variety of benefits, biostimulants may enhance fertilizer use efficiency, enhance tolerance to nutrients, water and salinity stress (Sci. Hort. 2015, 196, 3–14). The main aim of this project will be the production of algal extracts from different species of seaweeds, using mainly novel extraction techniques and analysis of the utilitarian properties of these extracts in germination tests and pot experiments. The best candidate/candidates as a biostimulant of plant growth will be selected.

Hypothesis 2. Seaweeds have very good biosorption properties and can be used in wastewater treatment and soil bioremediation: Pollution with toxic metals and its implications for public health and the environment has led to the increased interest in developing environmental biotechnology approaches. For this reason, a biosorption process can be used. This process relies on a passive cation binding by dead biomass (it's not metabolically controlled) and is based on the physicochemical interactions between metal ions in an aqueous solution and functional groups of the biosorbent's cell wall. This process is cheap and very efficient and can offer an alternative to the conventional methods used for the environment decontamination. Moreover, marine seaweeds are known to have very good biosorption properties and they can efficiently remove toxic metals from polluted soil and water. The main aim of this step will be the selection of different species of green, brown and red seaweeds and examination of their biosorption properties. The best candidate/candidates as a sorbent of toxic metal ions will be selected.

Principal scientific bottlenecks: The main obstacle for industrial markets is the access to large quantities of active molecules from available biomass. However, marine algae from the French Brittany and Poland coasts constitute a significant and diversified natural vegetable production. As a result, nearly 700 species of algae are currently listed on the Breton littoral zone and 40 species on the Polish littoral zones. Among the flora encountered in Brittany and in Mexico, native or introduced macroalgae can become **invasive or proliferative** and have profound adverse ecological impacts including the alteration of the ecosystem structure, the reduction of indigenous biodiversity, and economic losses. **In France**, strandings of *Solieria chordalis* (Gigartinales, *Solieriaceae*), *Ulva* sp. (Chlorophyta, *Ulvaceae)* and *Sargassum muticum* (Phaeophyta, Fucales) appear along the shallow sandy bays. Monthly monitoring of the (bio)chemical composition of *Ulva*, *Solieria chordalis* and *Sargassum muticum* has been conducted over the last 4 years. Several biochemical parameters were regularly measured bringing enhanced knowledge of the composition of this biomass and the breakdown into its primary metabolites. **In Poland**, green macroalgae dominate in the floral composition, especially *Enteromorpha ahlneriana*, *E. intestinalis*, *Cladophora albida*, *Cl. glomerata*, *Cl. rupestris*, *Cl. sericea*, *Cl. vagabunda*. Among brown algae – *Pilayella litoralis*, *Fucus vesiculosus* are distinguished. *Polysiphonia violacea*, *Ceramium diaphanum* are the main red algae and *Zostera marina* is a main vascular plant.

Unfortunately, there is a remarkable lack of detailed data about the processes of algal extraction technologies for agricultural purposes, mostly because the manufacturing methods are rarely published and held as proprietary information. In fact, several extraction procedures have been adopted for agricultural biostimulant production from marine macroalgae. In most cases, extracts are made by processes using water, alkalis or acids, or physically by disrupting the seaweed by low temperature milling to give a micronized suspension of fine particles. For seaweed utilization as fertilizer and biostimulant components, water extraction seems to be the most cost-effective and practicable tool for better release of micro- and macro-elements from the biomass. Thus, several reports described the use of water or alkaline extracts from algae as plant growth biostimulants (tomato, *Arabidopsis*, spinach, *Vigna sinensis*, etc.) under normal and stressed environments. In almost all cases, beneficial effects on growth of cereals, pulses, and flowering plants have been reported (Plant Soil, 2014, 383, 3–41; J. Appl. Phycol. 2014, 26, 465–490; J. Plant Growth Regul. 2009, 28, 386–399). Algal fertilizers are usually prepared with a chemical method where potash lyse and high temperature are applied. However, such conditions are quite severe and cause decomposition of most of the biologically active compounds. Thus, the primary challenge when extracting algal bioactive compounds will be to find a compromise between the cost of production of sufficient quantities and quality of compounds in the shortest timeframe, finding the optimum processing condition and meeting the principles of green chemistry and green technology (J. of Agric. Food Chem., 2013, 61: 4667–4675). In this context, the effort will be put on cell wall structure disruption in order to enhance the liquefaction of the seaweeds, the release of their internal components and their partial conversion.

The aim of this project is to investigate technical possibilities of using the marine macroalgae accumulating on the coasts and beaches to produce bioactive fractions for agricultural applications. Using combinations of innovative extraction processes, i.e. Enzyme Assisted Extraction (EAE), Ultrasounds Assisted Extraction (UAE), Pulsed Electric Fields (PEF), Microwave Assisted Extraction (MAE) alone or in combination for maximizing the liquefaction of the algal biomass. These processes could be combining with mechanic processes (extrusion, ball milling). The production of bioactive products or extracts at an industrial level is targeted within the concept of biorefinery. This bio-based technology also assumes the utilization of wasted seaweed biomass and post-extraction residues as sorbents of metal ions.

5.2 – Methodology Approach

1. *Production of seaweed extracts using* **combinations of innovative extraction processes** *i.e.* Enzyme Assisted Extraction (EAE) *with commercial enzymes* and/or water extraction will be evaluated in small reactors (from 1 to 6l). The resulting two phases (soluble & insoluble) will be quantified and qualified with a particular focus on biological activities into the soluble one. The soluble phase will be tested as a biostimulant. The insoluble phase can be resubmitted to additional treatment in order to check its biosorption properties. We can compare them with the raw biomass before extraction. For each case, the influence of key parameters on the extraction yield will be investigated such as the nature of enzyme(s) and different enzyme combinations. The PEF will be studied in collaboration with IRDL (UBS), MAE (time, temperature parameters) and UAE in WUST combining with EAE and mechanic processes (extrusion, ball milling).

2. *Analysis of extracts:* Resulting extracts will be biochemically characterized: dry matter content, mineral content, nitrogen content (Kjeldhal), peptide molecular size (HPLC-MS/MS), amino acids profile (HPLC-UVDAD), total sugars, uronic acids, sulfate groups, monosaccharides (HPAEC) and oligosaccharides molecular size profile (HPSEC–RI).

3. *Biostimulant properties of seaweed extracts:* The produced seaweed extracts will be tested in germination tests according to the International Seed Testing Association (ISTA). Germination tests will be evaluated using Jacobsen germinator on Petri dishes with model plants such as for example garden cress and/or radish. Different doses of seaweed extracts will be tested. Seaweed extracts are usually most efficient (stimulation of plant growth) in low concentrations. Algal extracts can be applied in three different methods:

- to the seeds (soaking of seeds in extract),
- foliar application (when seeds germinated),
- soil application (extract added to the Petri dish before seeds sowing).

In this study, **germination energy** (speed at which the seeds germinate, sometimes expressed as a percentage of the seeds germinated within the first week of analysis with respect to overall germination) and **germination percentage** (after the end of the experiment) will be evaluated. The effect of extracts on plant height, fresh and dry mass, chlorophyll content (SPAD equipment and spectrophotometric method) and root length will be examined. Experiments will be performed in four replications in Petri dishes, under standardized conditions – isolated box with adjustable lighting and temperature (temperature fluctuations ± 4°C) – Jacobsen apparatus. Each dish will be watered with the appropriate volume of algal extract, and the control group will be watered with the same volume of distilled water. The experiment will last for 12 to 14 days. The best concentration of algal extract can be later tested in pot experiments. The same parameters of plants, as in germination tests will be examined. It will last also about 12-14 days. The obtained results will be elaborated statistically, using STATISTICA software. Descriptive statistics (average, standard deviations) for all experimental groups will be performed. The normality of the distribution of experimental results will be assessed by the Shapiro–Wilk test and the homogeneity of variances by the Brown & Forsythe's test. On this basis, the statistical test used to investigate the significance of differences between the tested groups will be selected. The differences between the two groups will be investigated with a *t*-test and between several groups with the one-way analysis of variance (ANOVA) using the Tukey multiple comparison test (for normal distribution and the homogeneity of variances). In the case of the lack of the normal distribution, the Mann-Whitney test will be used (for two groups) and the Kruskal–Wallis test (for more than two groups). The results will be considered significantly different when $p<0.05$.

4. *Utilization of a raw biomass of seaweeds as a biosorbent of toxic metals*: The biomass of red/brown and green seaweeds, as well as post-extraction residue from for example anenzyme/water extraction will be used as a sorbent for soil and/or water bioremediation. Biosorption properties of collected seaweeds will be tested on Cr(III) ions – chosen as a model ion (determination of the concentration in aqueous solutions by spectrophotometric method).

The experiments will involve the choice of the best experimental conditions for the biosorption process – e.g., biosorbent size (sieve analysis, e.g., 500, 200 μm), pH (3, 4, 5), initial metal ion concentration (e.g., 100, 200 and 300 mg/l), biomass content in the solution (e.g., 1g/l; 2 g/l, 5 g/l). For the biosorption process, kinetic and equilibrium experiments will be performed for the biomasses and post-extraction residue from extraction of seaweeds. These studies will be carried out in Erlenmeyer flasks, which contain solutions with chromium ions prepared by dissolving in deionized water respective weighed amounts of inorganic salts ($Cr(NO_3)_3 \cdot 3H_2O$). The entire system will be thermostated and stirred with shaking at a constant stirring rate. During kinetic studies, the samples of the biomass in solution with Cr(III) ions will be collected at appropriate time intervals in order to determine the concentration of a metal ion during the biosorption process and to establish the time necessary to reach the state of equilibrium. It is necessary to conduct a study of equilibrium of biosorption process. The obtained results will be described by appropriate models. Mechanism of this process will be studied. The biomass of seaweeds before and after biosorption process will be examined using FTIR technique, in order to establish which functional groups on the surface of the algal cell wall participated in the biosorption process.

6. Collaborations during the project

Co-tutelle project: Wroclaw University of Science and Technology,Faculty of Chemistry, Department of Advanced Material Technologies, Wroclaw, Poland

In collaboration with **Universitas Negeri Makassar (Indonesia)** and Cereal Crops Research Institute, Allepolea, we have the possibility to study the influence of our extracts on the mineral composition of the soil by measuring greenhouse gas production and to determine the impact of extracts on maize crop growth and soil quality. (PHC Nusantara 2019-2020). They are studying crop enhancement by soil emulsification and the increase of fertility by means of protein or nitrogen (NH_4^+ or NO_3^-). They would also like to analyze the emission of N_2O gases that are released from the nitrification-denitrification process (with Corn (*Zea mays*) as a model). They are using a maize field at pilot-scale (size 10x15 m^2).

IRDL Institut de Recherche Dupuy de Lôme (UBS) Pr. Jean-Louis Lanoisellé : Pulsed electric field assisted extraction of intracellular compounds from macroalgae

7. Candidate

Autonomy, serious, Good knowledge in Phycology, Ecofriendly extraction processes, biochemistry, Plant physiology, English