



## No Detectable Toxicity of Marine-Based Nutraceuticals for Human Cells

Sylvain Le Coguic<sup>1</sup>, Gilles-Éric Seralini<sup>2\*</sup>

<sup>1</sup>Laboratoire Biothalassol Duchange, ZA de la Hogue, 1 rue de la Plaine, Benouville, France

<sup>2</sup>University of Caen Normandy, Network on Risks, Quality and Sustainable Environment, Esplanade de la Paix, Caen Cedex, France

\*Corresponding author: Gilles-Éric Seralini, University of Caen Normandy, Network on Risks, Quality and Sustainable Environment, Esplanade de la Paix, CS 14032, Caen Cedex 5, France. Tel: +33231565489; Email: gilles-eric.seralini@unicaen.fr

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### Abstract

Marine food supplements, also called marine-based nutraceuticals, represent an innovative trend in the food supplements. They may be claimed to prevent or cure chemical intoxications. Few of these are tested *in vitro* on human cells, including for cytotoxicity tests, as a first step before characterization of their mechanisms of action *in vivo* and for their cellular impacts. Here we tested *in vitro* on human cells 11 marine-based food supplements with salts and marine or terrestrial plant extracts prepared by two laboratories. The tests included a cytotoxicity assay (MTT assay) and a proliferation assay (BrdU incorporation) with JEG-3, HepG2, and HEK293 human cell lines. While the recommended dose is 2% for most of these compounds, the range of dilution tested was 0.1% to 100%. None of the marine food supplements at physiological concentrations exerted any side-effect observable in these conditions, on the three selected human cell lines, except to a slight extent Mer<sup>®</sup>Emincyl on JEG-3 only. Their detoxification capacities should now be investigated.

**Keywords:** Bioactive Molecules; Cytotoxicity; Food Supplements and Health; Marine Nutraceuticals

### Introduction

Recent trends in functional foods and supplements have demonstrated that bioactive molecules play a major therapeutic role in human disease. Nutritionists and biomedical and food scientists are working together to discover new bioactive molecules that have increased potency and therapeutic benefits. Marine life constitutes almost 80% of the world biota, with thousands of bioactive compounds and secondary metabolites derived from marine invertebrates such as tunicates, sponges, molluscs, bryozoans, sea slugs, and many other marine organisms. These bioactive molecules and secondary metabolites possess antibiotic, antiparasitic, antiviral, anti-inflammatory, antifibrotic and anticancer activities [1]. They are also inhibitors or activators of critical enzymes and transcription factors, competitors for transporters, and sequestrants that modulate various physiological pathways [1].

Few of these have been tested *in vitro* on human cells, including for cytotoxicity tests. As a first step before characterization of their

mechanisms of actions *in vivo* and for their cellular impacts, we tested 10 marine-based food supplements *in vitro* on human cells. The tests included a cytotoxicity assay and a proliferation assay. We used the embryonic (HEK 293), placental (JEG-3), and young adult hepatic (HepG2) human cell lines because they are well characterized and validated as useful models to test toxicities of chemicals [2-4], corresponding to what is observed on fresh tissue or primary cells [5-7]. In particular, HepG2 cells have been shown to be a validated model for assessing the hepatotoxic properties of a product [8], since phase I and II enzymes are active within the cells [9,10]. These marine-based food supplements also comprise medicinal plant extracts. The recommended dose is 2% for most of these compounds, but the range of dilution we tested was 0.1% to 100%. None of them was toxic at physiological concentrations.

### Results

All the marine food supplements (Table 1) were tested for cytotoxicity (MTT assay) and/or proliferative effects (BrdU incorporation assay) on human cell lines. When both tests were used simultaneously (for Plasma Eau De Mer Isotonique and Plasma Eau De Mer Hypertonique), they gave similar results.

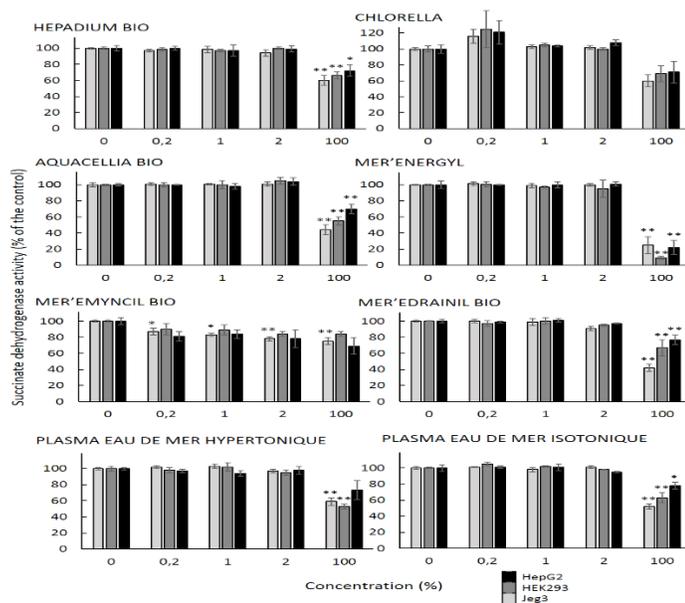
Commercial Product Name	Detailed Composition
HEPADIUM BIO	Sea water, <i>Betula sp.*</i> sap, <i>Desmodium sp.</i> , <i>Himantalia elongata</i> , <i>Silybum marianum*</i> , <i>Rosmarinus officinalis*</i> , <i>Raphanus sativus niger*</i> , <i>Ascophyllum sp.*</i> , <i>Citrus limon*</i> juice, <i>Euterpe oleracea*</i> powder
CHLORELLA	<i>Chlorella pyrenoidosa</i>
AQUACELLIA	Sea water, <i>Fucus sp.*</i> , <i>Salicornia europea*</i> , <i>Beta vulgaris ssp. maritima*</i> , <i>Orthosiphon aristatus*</i> , <i>Raphanus sativus niger*</i> , <i>Filipendula ulmaria*</i> , <i>Sambucus nigra*</i> , <i>Juniperus communis*</i> , <i>Citrus limon*</i>
MER'ENERGYL	Sea water, 76% richer in Mg than Plasma Sea Water hypertonic
MER'EMYNCIL BIO	<i>Foeniculum vulgare*</i> juice, <i>Ananas comosus*</i> concentrate, sea water, <i>Citrus aurantium*</i> dry extract, <i>Citrus limon*</i> concentrate juice, <i>Curcuma longa*</i> powder, <i>Pilosella officinarium*</i> , <i>Fucus sp.*</i> , <i>Viola tricolor*</i> , <i>Sambucus nigra*</i> flowers, <i>Fraxinus excelsior*</i> leaves, <i>Glechoma hederacea*</i> , <i>Rosmarinus officinalis*</i> leaves, <i>Levisticum officinale*</i> leaves, <i>Taraxacum sp.*</i> leaves, <i>Ananas comosus</i> , water
MER'EDRAINIL BIO	Sea water, <i>Fucus sp.*</i> , <i>Illicium verum*</i> , <i>Arctium sp.*</i> roots, <i>Cynara scolymus*</i> leaves, <i>Juniperus communis*</i> seeds, <i>Foeniculum vulgare*</i> seeds, <i>Thymus vulgaris*</i> leaves, <i>Taraxacum sp.*</i> , <i>Aloe vera*</i> , <i>Raphanus sativus niger*</i> juice, <i>Citrus limon*</i> concentrate juice, <i>Chlorella pyrenoidosa</i>
PLASMA SEA WATER HYPERTONIC (EAU DE MER HYPERTONIQUE)	Sea water from North Brittany at a depth below 20 meters, in the Archipelago of Chausey, in an ecologically protected area classified as Natura 2000, with a cold microfiltration with adapted Pall cartridges to preserve probiotic properties and to avoid bacteria, salts 35 g/l (Biothalassol product)
PLASMA SEA WATER ISOTONIC (EAU DE MER ISOTONIQUE)	Sea water, see above (Biothalassol)
SERUM OCEANIC	Sea water, salmon DNA, potassium sorbate, sorbate de potassium, essential oil of <i>Citrus limon*</i> zest
QUINTON ISOTONIC	Sea water isotonic (Product on the market, for comparison)
QUINTON HYPERTONIC	Sea water hypertonic (Product on the market for comparison)
*organic products	

**Table 1:** Detailed name and composition of the marine food supplements assayed.

### Cytotoxicity assay

The toxicity threshold of each product was determined by MTT using 5 concentrations on JEG-3, HEK 293 and HepG2 human cell lines (Figure 1). The lowest concentration exerting a significant toxic effect was considered to be the toxicity threshold.

Above 0.2% in one of the 3 cell lines, only Mer'Emincyl Bio was slightly but significantly cytotoxic (13% decrease in viability). Except for this case, all the products tested were not cytotoxic up to 2%. At 100%, cells exposed to the pure raw product underwent a variable viability decrease, probably due to the osmotic shock or the lack of other nutrients. 100% toxicity was never reached.

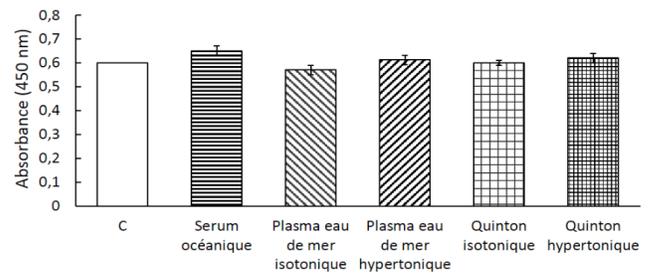


**Figure 1:** Effects of various marine food supplements on viability in three human cell lines.

Effects on the mitochondrial Succinate Dehydrogenase (SD) activity reflecting cell respiration are shown in comparison to the control (100%) in serum-free medium after 24h exposure. The marine food supplements are detailed in Table 1. Concentrations are in % (recommended dilution 2% in water). All data are mean  $\pm$  standard error of the mean (SEM). All the experiments were repeated 3 times in triplicates. Cell lines are HepG2 (black columns), HEK 293 (dark grey) and JEG-3 (clear grey). The three last compounds of Table 1 gave essentially similar results as “Plasma Eau de Mer” (name of the product (Pure, non-polluted Sea Water)).

### Proliferation assay

The effect of Serum Oceanic, Plasma Sea Water Isotonic, Plasma Sea Water Hypertonic, Quinton Isotonic and Quinton Hypertonic on cell proliferation was then assessed by following BrdU incorporation (Figure 2). HepG2 cells were treated with 2% of each product for 24h. Plasma Sea Water Isotonic and Plasma Sea Water Hypertonic had no effect on cell proliferation, as it was the case for cell viability. Serum Oceanic, Quinton Isotonic and Quinton Hypertonic also had no effect on cell proliferation in comparison to control.



**Figure 2:** Effects of various marine food supplements on proliferation in HepG2 human cell lines.

BrdU incorporation into the newly synthesized DNA of replicating cells was assessed using a monoclonal anti-BrdU antibody followed by colorimetric detection at 450 nm. The marine food supplements are detailed in Table 1 and tested at the recommended dilutions of 2%. All data are mean  $\pm$  Standard Error of the Mean (SEM). All the experiments were repeated 3 times in triplicates. C: Control.

### Discussion

None of these marine food supplements had any observable physiological or biochemical impact at 2%, on all parameters measured, except Mer’Emincyl Bio, which exerted only a 20% negative effect on JEG-3 cells. The cytotoxicity observed at the non-physiological dose of 100% may be explained by an osmotic shock, or more probably by a lack of essential nutrients for the cell growth without the cell medium. The observance of a lack of toxicity *in vitro* is a first step before the assessment of benefit properties and investigation of the mechanisms of action, both *in vivo* and *in vitro*.

Most of the products tested here are mixtures of algae and other plant extracts. Algae provide health-beneficial compounds such as polysaccharides (carrageenan, agar agar, fucans and fucanoids), antioxidant phenolic compounds (phlorotannins) and pigments (carotenoids), vitamins, and minerals [1]. For instance, the genus chlorella (the component of Chlorella is also present in Mer’Edrainyl Bio) contains high levels of carotenoids, phenolic compounds and tocopherols, contributing to the antioxidant properties of this microalga [11]. *Himantalia elongata* (contained in Hepadium Bio) also displays a high polyphenolic concentration, leading to antioxidant properties. The nutritional composition of this brown alga includes high levels of total dietary

fiber and essential amino acids, and while the level of lipids is low, the polyunsaturated fatty acid content is high [12]. The anti-inflammatory capacity of *Fucus sp.*, contained in Mer'Edrainil Bio and Mer'Emincyl Bio, has been well characterized [13,14], among several other beneficial effects [15]. However, the slight toxic effect of Mer'Emincyl Bio on JEG-3 cells, the most sensitive of the 3 cell lines as previously measured [16], may be due to the draining and slimming properties of this product due to the leaf extracts from *Taraxacum sp.* [17] and *Fraxinus excelsior* [18] that it contains.

The originality of the food supplements tested in this study lies in the fact that these algae extracts are mixed with organic plant extracts and that these mixtures are further diluted in sea water. The benefits for human health of all the plants included in these different products are numerous. Among others and of special interest, flavonoids are nearly ubiquitous in plants but particularly rich in citrus fruits [19], and *citrus limon* is a common ingredient of 5 of the 11 products tested here. The flavonoids have long been recognized to possess anti-inflammatory, antioxidant, anti-allergenic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities, which are reviewed [20]. For these reasons, certain flavonoid-rich plants and spices have long been used in traditional medicine. Specifically, the anticancer, cardiovascular and anti-inflammatory properties of citrus flavonoids have been extensively discussed [21]. All the products tested here contain sea water as a solvent, and/or contain mother liquors rich in magnesium salts; 5 products are exclusively composed of high-quality deep-sea water. It has been shown that deep-sea water provides intestinal protection [22], modulates blood pressure and exhibits hypolipidemic effects [23], and improves cardiovascular hemodynamics [24].

As a follow-up for the *in vitro* experiments, and since most of the individual compounds of these marine food supplement possess antioxidant activities, it would be interesting to assess the antioxidant properties of these mixtures. This could be performed *in vitro*, by measuring the Nitric Oxide (NO) synthesis in cells co-exposed to an oxidant chemical. *In vivo*, the oxidative stress index could be measured in high fructose and high-fat-fed rats. For this purpose, the levels of specific markers could be measured in the plasma, liver and kidney, as well as the level of reduced glutathione and the activities of antioxidant enzymes, such as glutathione peroxidase and catalase in the blood.

## Materials and Methods

### Chemicals and marine food supplements/nutraceuticals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was prepared as a 5 mg/mL stock solution in phosphate-buffered saline (PBS), filtered through a 0.22 µm filter before use, and diluted to 1 mg/mL in a serum-free medium. MTT was obtained from DM Labo (Caen, France). Marine food

supplements names and compositions are reported in Table 1. They were prepared and provided by Biothalassol Duchange Laboratory (Benouville, France) and Alderney Laboratory (Caen, France).

### Cell lines and treatments

The human embryonic kidney 293 cell line (HEK 293, ECACC 85120602) was provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). The hepatoma cell line HepG2 was provided by ECACC (85011430). The JEG-3 cell line (ECACC 92120308) was provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM (Abcys, Paris, France) containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (a mixture of penicillin, streptomycin and fungizone) (Lonza, Saint Beaulieu, France), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France) and 10% Fetal Bovine Serum (PAA, Les Mureaux, France). JEG-3 cells were supplemented with 1 mM sodium pyruvate. Cells were grown with this medium at 37 °C (5% CO<sub>2</sub>, 95% air) during 48 h to 80% confluence, then washed and exposed 24 h with serum-free EMEM to all the marine food supplements. Before treatment, all the marine food supplements were diluted in serum-free medium and adjusted to a similar pH. This model has been previously validated [25], in that toxic effects were similar in the presence of serum but delayed by 48 h.

### Cytotoxicity biomarkers

After 24 h, cells at 80% of confluence were washed with serum-free EMEM and then exposed to various concentrations of marine food supplements in EMEM serum-free medium for 24 h. After treatments, a Succinate Dehydrogenase (SD) activity assay (MTT) [26] was applied, as described previously [27]. The integrity of mitochondrial dehydrogenase enzymes indirectly reflects cellular mitochondrial respiration. Briefly, the MTT reagent (thiazolyl blue tetrazolium bromide, Sigma Aldrich, Saint-Quentin Fallavier, France) was added to cells and the optical density of the isopropanol (with 0.04 N of chlorhydric acid)-dissolved formazan salts was measured after a 3 h incubation. The percentage of surviving cells is expressed as the ratio of optical density of treated cells versus untreated cells (control). The optical density was measured at 570 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France).

### Cell proliferation assay

The cell proliferation was measured through a bromodeoxyuridine (BrdU) incorporation assay: the colorimetric BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, UK) was used. HepG2 cells were seeded in 24-well plates (30,000 cells/well) and allowed to attach overnight. Cells were then treated for 24 h with different marine food supplements at 2% in triplicate wells per condition. BrdU was added 4 h before the end of the incubation period. Cells were then fixed, DNA was denatured, and

BrdU content was then assessed using a monoclonal anti-BrdU antibody, following the manufacturer's instructions.

### Statistical analyses

All data were presented as the mean  $\pm$  Standard Error of the Mean (SEM) in scatter plots. Statistics were performed using GraphPad Prism 5 (GraphPad software, La Jolla, USA) software. Data were checked for Gaussian distribution by a Shapiro test and for homoscedasticity (Barlett's test). In cases where these two conditions were met, the multiple comparisons test was an ANOVA followed by Bonferroni post hoc test. In the other cases, statistical differences were determined by a non-parametric Kruskal-Wallis test followed by a Dunn's post hoc test for multiple comparisons. Significant levels were reported with  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*)

### Conclusions

None of the marine food supplements exerted any observable side-effect in these conditions on the three selected human cell lines. This is the first demonstration of lack of cytotoxicity of these marine food supplements at a physiological level and a first step before characterization of their mechanisms of actions *in vivo*, potential detoxication capacities, and cellular impacts.

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### Author Contributions

GES conceived the study, designed the work and directed the preparation of the manuscript. Sylvain Le Coguic helped to develop the products and prepared them. Both authors read and approved the final manuscript.

### Conflicts of Interest

The researchers from the University of Caen Normandy (GES) in charge of the assessment of the marine food supplements declare that they have no financial or other interests in the commercial development of these products. The development of the products (SLC) was performed completely independently of the present assessment.

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