BIOATLANTIS OMRI PETITION – *Laminaria species* and *Ascophyllum nodosum* SEAWEED EXTRACTS AS SYNTHETIC SUBSTANCE FOR USE IN ORGANIC CROP PRODUCTION

Item A

Synthetic substances allowed for use in organic crop production, § 205.601 of NOP.

Item B

1. **Substance's Chemical Name** – Brown seaweed (Phaeophyceae class) extract.
2. **Manufacturer's Data** –
   Company Name: BioAtlantis, Ltd.
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3. **Intended Use** – Brown seaweed (Phaeophyceae class) extract (namely from *Laminaria species* or *Ascophyllum nodosum*) is intended to work as plant strengthener for use in various fruits, vegetables, and cereal crops.

4. **A list of the crop, livestock or handling activities for which the substance will be used. If used for crops or livestock, the substance's rate and method of application must be described. If used for handling (including processing), the substance's mode of action must be described.**

Brown seaweed (phaeophyceae class) extract (namely from *Ascophyllum nodosum*) for application in:

Cereals such as (but not limited to) maize to improve seed germination (Sharma *et al.*, AFBI, 2009, unpublished data, see Appendix I) and barley to improve shoot growth (Sharma *et al.*, AFBI, 2008, unpublished data, see Appendix I).

Grasses species such as (but not limited to) perennial ryegrass & creeping bentgrass to increase root growth (Fleming *et al.*, AFBI, 2009, unpublished data, see Appendix I).

Broad acre crops such as (but not limited to) to oilseed rape for improving root and shoot growth (Jannin *et al.*, 2013, see Appendix I).

Rate and method of application: Ecolicitor: 1.5 to 2.5 L /ha diluted in 500L of water, foliar application or fertigation.

Brown seaweed (phaeophyceae class) extract (namely from *Laminaria species*) for application in:

Broad acre crops such as (but not limited to) barley and maize to improved seedling emergence, (Mercer *et al.*, 2010, see Appendix I).

Rate and Method of application:

AgriPrime Nematec: 2.5L/ha diluted in 250L of water, foliar application or fertigation.

5. **Source of the Substance and its Processing** – Brown seaweed extract (*Laminaria species* and *Ascophyllum nodosum*) is attained in a sequential simple process:
   Step 1 – Fresh *Laminaria species* or *Ascophyllum nodosum* seaweed, harvested on Ireland, is extracted with tap water whose pH is lowered (to a 3.5 minimum) by addition of sulfuric acid in a low concentration of less than 980 ppm. This acid acts as a processing aid within this step only.
   Step 2 – The extraction mixture is centrifuged for the separation of the seaweed insoluble materials from the liquid extract.
   Step 3 – The liquid extract's pH is adjusted to a pH near neutral by addition of potassium hydroxide.
   This process as carried out by BioAtlantis Ltd. yields a greenish (*Laminaria species*) or brownish (*Ascophyllum nodosum*) liquid with a typical marine odour.
6. **Substance Reviews by State/Private Certification Programs or other Organizations** – There is a petition for laminarin, a substance extracted from *Laminaria species* seaweed with the use of sulfuric acid, currently under review by the NOSB, and which has received a preliminary favourable review by the Crops Subcommittee (see recommendation as Appendix II).
   Moreover, the U.S. Food and Drug Administration (FDA) has expressed the view that a *Laminaria japonica* (species similar to *Laminaria species*) extract can be put in the category of "Generally Recognized as Safe" (GRAS) substances (see Appendix III), in accordance to scientific procedures. The competent FDA committee concluded that there was no substantive evidence or reason to suspect a significant risk to public health from use of brown algae ingredients in foods.
7. **Regulatory Authority Registrations –** The substance (brown seaweed extracts) has already been registered with different state regulatory authorities:
   - *Environmental Protection Agency (EPA):* Internal tracking number for *Laminaria species* E17100793. It is an approved substance by EPA.
   - *Environmental Protection Agency (EPA):* Internal tracking number for *Ascophyllum nodosum* E17130790. It is an approved substance by EPA.
8. **Chemical Abstract Service (CAS) Number and Product Labels** – BioAtlantis Ltd. prepares two main brown seaweed (Phaeophyceae class) extracts. *Ascophyllum nodosum* extract: 84775-78-0 and *Laminaria species* extract: 90046-12-1. The product labels are presented in Appendix IV.
9. **Substance's Physical Properties and Chemical Mode of Action** – BioAtlantis Ltd. prepares two main brown seaweed (Phaeophyceae class) extracts, whose physical properties are quite similar.
   Laminaria species seaweed extract is characterized by the following properties:
   General physical properties – It is a greenish liquid with marine odour, a specific gravity of approximately 1.2, and a solubility higher than 99 %.
   General chemical characterization – It presents a pH range of 3.5-5.5 (as intended for BioAtlantis Ltd. products), 25-30 %, w/w, organic matter, and 10-15 %, w/w, inorganic matter.
   (a) Chemical Interactions with other Substances – It does not react strongly with acid or alkaline substances. Its reactivity with oxidants and reducing substances is also limited. It can also be mixed with other substances for crop protection provided that these are not

too sensitive to a pH below neutral. Miscibility with other liquid substances either aqueous or non-aqueous is high. There is no enhanced reactivity with metals regardless of the pH and ionic strength conditions. There is neither flammability nor explosiveness potential.

(b) *Toxicity and Environmental Persistence* – No toxicity has been found in *Laminaria species* extracts and its components do not present environmental persistence issues. Indeed, the components of these extracts are of low toxicity and break down quickly in the environment. The only area of concern would be some environmental organic residues in seaweeds, but even this present no major environmental problem (Appendix V).

(c) *Environmental Impacts from its Use and Manufacture* – The *Laminaria species* seaweed extract is manufactured in Ireland and no significant environmental impacts have been detected until now. Moreover, there are no known deleterious environmental impacts arising from the use of this substance.

(d) *Effects on Human Health* – The *Laminaria species* seaweed extract presents virtually no toxicity to humans. These extracts from this brown seaweed species are not considered hazardous to human health (Appendix VI).

Effects on Soil Organisms, Crops, or Livestock – No adverse effects on soil organisms or livestock are anticipated. The *Laminaria species* seaweed extract is expected.to increased performance, root and shoot growth and to improve soil microbial count.

Ascophyllum nodosum seaweed extract is characterized by the following properties:

General physical properties – It is a brownish liquid with marine odour, a specific gravity of approximately 1.2, and a solubility higher than 99 %.

General chemical characterization – It presents a pH range of 3.5-5.5 (as intended for BioAtlantis Ltd. products), 10-20 %, w/w, organic matter, and 5-15 %, w/w, inorganic matter.

(e) *Chemical Interactions with other Substances* – It does not react strongly with acid or alkaline substances. Its reactivity with oxidants and reducing substances is also limited. It can also be mixed with other substances for crop protection provided that these are not too sensitive to a pH below neutral. Miscibility with other liquid substances either aqueous or non-aqueous is high. There is no enhanced reactivity with metals regardless of the pH and ionic strength conditions. There is neither flammability nor explosiveness potential.

(f) *Toxicity and Environmental Persistence* – No toxicity has been found in *Ascophyllum nodosum* extracts and its components do not present environmental persistence issues. Indeed, the components of these extracts are of low toxicity and break down quickly in the environment. The only area of concern would be some environmental organic residues in seaweeds, but even this present no major environmental problem (Appendix V).

(g) *Environmental Impacts from its Use and Manufacture* – The *Ascophyllum nodosum* seaweed extract is manufactured in Ireland and no significant environmental impacts have been detected until now. Moreover, there are no known deleterious environmental impacts arising from the use of this substance.

(h) *Effects on Human Health* – The *Ascophyllum nodosum* seaweed extract presents virtually no toxicity to humans. These extracts from this brown seaweed species are not considered hazardous to human health (Appendix VII).

(i) *Effects on Soil Organisms, Crops, or Livestock* – No adverse effects on soil organisms or livestock are anticipated. The *Ascophyllum nodosum* seaweed extract is expected to improve plant growth, marketable grade and seedling emergence.

10. **Safety Information about the Substance** – Material Safety Data Sheets (MSDS) for *Laminaria species* as well as *Ascophyllum nodosum* are attached to this main document (Appendix VI & Appendix VII). Substance reports for both brown algae extracts by the National Institute of Environmental Health Sciences (NIEHS) are not available.
11. **Research Information about the Substance** – The available research information concerning brown seaweed (Phaeophyceae class) extracts, *Laminaria species* and *Ascophyllum nodosum*, can be divided in the following categories:
    - Chemical and other properties of the main components of seaweed extracts:
      Bacic, A.; Fincher, G. B.; & Stone, B. A. (2009). Chemistry, biochemistry, and biology of 1-3 beta glucans and related polysaccharides. Academic Press: Burlington, U.S.A.
      Black, W. A. P.; & Dewar, E. T. (1949). Correlation of some of the physical and chemical properties of the sea with the chemical constitution of the algae. Journal of the Marine Biological Association of the United Kingdom, 28(3), 673-699.
      Li, B.; Lu, F.; Wei, X.; & Zhao, R. (2008). Fucoidan: Structure and bioactivity. Molecules, 13, 1671-1695.
    - Utilization of sulfuric acid in the substance's preparation process:
      Bassi, R.; Prasher, S. O.; & Simpson, B. K. (2000). Extraction of metals from a contaminated sandy soil using citric acid. Environmental Progress, 19(4), 275-282.
      Kpomblekou, A. K.; & Tabatabai, M. A. (1994). Effect of organic acids on release of phosphorus from phosphate rocks1. Soil Science, 158(6), 442-453.
    - Health impacts and toxicological information:
      Gupta, S.; & Abu-Ghannam, S. (2011). Bioactive potential and possible health effects of edible brown seaweeds. Trends in Food Science and Technology, 22(6), 315-326.
      Wang, T.; Jónsdóttir, R.; & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chemistry, 116(1), 240-248.
      Yuan, Y. V.; & Walsh, N. A. (2006). Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds. Food and Chemical Toxicology, 44(7), 1144-1150.
    - Environmental impacts:
      Verkleij, F. N. (1992). Seaweed extracts in agriculture and horticulture: A review. Biological Agriculture & Horticulture: An International Journal for Sustainable Production Systems, 8(4), 309-324.
    - Fate of seaweed extract components in the environment:
      Chesters, C. G. C.; & Bull, A. T. (1963). The enzymic degradation of laminarin. 1. The distribution of laminarinase among micro-organisms. Biochemical Journal, 86(1), 28-31.
    - Effect on Crops and their mode of action:
    - Sharma S et al. (2009). Germination of Maize seeds following treatment with seaweed extracts. Agri-Food and Biosciences Institute (AFBI), Belfast, UK.
    - Sharma S et al. (2008). Assessment of seaweed extracts to improve cereal tolerance to drought stress. Agri-Food and Biosciences Institute (AFBI), Belfast, UK.
    - Fleming et al. (2009). Effects of seaweed extracts on the growth of creeping bentgrass and perennial ryegrass. Agri-Food and Biosciences Institute (AFBI), Belfast, UK.
    - Jannin, L.; Arkoun, M.; Etienne, P.; Laine, P.; Goux, D.; Garnica, M.; Fuentes, M.; San Francisco, S.; Baigorri, R.; Cruz, F.; Houdusse, F.; Garcia-Mina, J. M.; Yvin, J. C.; & Ourry, A. (2013). *Brassica napus* growth is promoted by *Ascophyllum*

nodosum (L.) Le Jol. Seaweed Extract: Microarray analysis and physiological characterization of N, C, and S Metabolisms. Journal of Plant Growth Regulation, 32(1), 31-52.

- Mercer, P. C.; Copeland, R. B.; Sharma, H. S. S.; & Bingham, J. (2010). Seaweed extracts as possible agents in improving the emergence of barley, oats and maize in Northern Ireland. Advances in Animal BioSciences, 1(1), 332.

12. **Petition Justification Statement**:

BioAtlantis Ltd. proposed substance, brown seaweed extract (prepared of only two species, *Laminaria species* or *Ascophyllum nodosum*), is composed of naturally occurring components extracted from seaweed, such as laminarin or fucoidan. Petitioned substance is to be considered as a synthetic substance allowed for use in organic crop production, as set in § 205.601 of NOP.

There is a natural gain of plant strength that could not be brought about by other organic means. Indeed, the components present in the petitioned substance are able to help in crop protection, given their plant health strengthening action, for instance, bioactives present in Laminaria based extract can modulate plant physiological processes towards improved plant health. This can be enormously beneficial to the organic plant growers worldwide, since it endows them with a natural means to increase plant strength without deploying other synthetic substances (see papers above in section **11. Research Information about the Substance**, under title "Effects on Crops and their Mode of Action"). In fact, the petitioner is not aware of any nonsynthetic substances, synthetic substances on the National List, or alternative agricultural methods that could be used instead of this natural mix of components found in the petitioned substance. This substance is non-toxic to mammals, birds, insects, and plants, does not bioaccumulate, and its biodegradation in the environment can proceed by several routes, thereby yielding constituents that can still be used by plants for their nutrition.

BioAtlantis Ltd. has developed brown seaweed (Phaeophyceae class) extracts from *Ascophyllum nodosum* and *Laminaria species* by a new pathway, which involves extraction without significant hydrolysis in mild acidic conditions (pH > 3.5). These conditions do not alter the chemical structure or bioactivity of the substance's components, such as laminarin and fucoidan. Therefore, the prepared brown seaweed extract does not undergo any chemical alteration, being its natural character wholly kept by the process. Furthermore, afterwards, a near neutral pH is established by addition of potassium hydroxide. This acidic extraction is required for maximizing the extraction of nonsynthetic components with a very positive impact on plant health and strength and minimizing the extraction of nonsynthetic components with negative effects on plants. In fact, acid operation prevents the simultaneous extraction of other compounds such as alginates, typically extractable at neutral pH.

For adjusting pH to acidic conditions, BioAtlantis Ltd. tested different acids and most effective was sulfuric acid, a synthetic substance. However, given its strength, addition of a small amount of this synthetic substance (< 980 ppm) to the extracting water is required. The low amount of sulfuric acid ensures a product with a high bioactive component concentration and reduces any potential associated chemical effects. Indeed, sulfuric acid does not participate or promote complexation and other reactions that may alter the optimal combination of bioactive components, as is the case of several organic acids, such as citric acid. The latter can extract undesirable components, such as metals, and there are scientific

studies showing that organic acids are more effective than sulfuric acid in releasing phosphate to liquid extracts (see papers above in section **11. Research Information about the Substance**, under title "Utilization of Sulfuric Acid in the Substance's Preparation Process"). Therefore, sulfuric acid provided for a maximal availability for plants of important seaweed bioactive molecules, namely, laminarin and fucoidan, with minimal risk of including deleterious components in the extract. Though sulfuric acid is not allowed in "(1) Aquatic plant extracts", it is allowed under the same broad category "(j) As plant or soil amendments" in subcategory "(7) Liquid fish products" of the § 205.601 of NOP. Moreover, the addition of potassium hydroxide completely eliminates any trace of sulfuric acid, yielding a maximum of 1740 ppm of potassium sulfate, which is totally dissociated in solution, given this salt's very high water solubility of 111 g/L at 20 °C. Such a low amount of sulfate in the typical dose rates to be used for the petitioned substance in agriculture (see above section **4. Handling/Processing Activities**) does not pose any phytotoxic effect and does not alter the fundamental nature and purpose of the substance.

This reasoning is further supported by the fact that BioAtlantis Ltd. will use sulfuric acid prepared from elemental sulphur, which is also set in a specific subcategory, "(2) Elemental sulfur", of "(j) As plant or soil amendments" and its use would be restrained to pH adjustment for ensuring the correct acidic pH during aqueous extraction. Hence, its role would be only to adjust pH and to ensure a final aquatic plant extract product with a pH in the 3.5-5.5 range.

Finally, it must be stressed that the inclusion of the brown seaweed (Phaeophyceae class) extract in the category § 205.601 of NOP is based on the fact that the preparation of this substance does not involve any chemical alteration of the bioactive components (laminarin, fucoidan, and others) of the seaweed. They are only removed from the seaweed cells to a liquid supernatant under mildly acidic conditions. Besides, the addition of sulfuric acid occurs at low levels and it is neutralised, rendering sulfate ions identical to those found naturally in living organisms. Therefore, BioAtlantis, Ltd. deeply believes in the appropriateness and fundamental advantage for the cause of organic agriculture that can be derived from the acceptance under § 205.601 of the brown seaweed extract substance by the National Organic Standards Board (NOSB).

13. **Confidential Business Information (CBI)–** None.

APPENDIX I (CITED STUDIES)

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Results of laboratory tests on the effects of seaweed extracts and other organic based materials on the growth of creeping bentgrass and perennial ryegrass.

Test 1: Effects on turfgrass root growth

Grass seedlings (x7 replication) were established in sand columns (USGA specification sand) and sprayed weekly (equivalent to 20 litres /Ha) with the appropriate product. Roots were analysed using WinRhizo after 25 days

Test concentrations:
All products used at 0.1% v/v except Fucoidan (0.1% w/v)

Table 1 Perennial ryegrass mean root length (mm)

	-Mm
Control	69.4[a]
Ecolicitor	**79.6[b]**
Fucoidan	129.2[c]
P1	114.8[c]
P2	62.1[a]

Within columns means sharing the same letter are not significantly different (LSD p<0.05)

Table 2 Creeping bentgrass mean root length (mm)

	-Mm
Control	17.4[a]
Ecolicitor	**22.8[b]**
Fucoidan	27.1[b]
P1	19.3[a]
P2	16.3[a]

Within columns means sharing the same letter are not significantly different (LSD p<0.05)

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Brassica napus Growth is Promoted by *Ascophyllum nodosum* (L.) Le Jol. Seaweed Extract: Microarray Analysis and Physiological Characterization of N, C, and S Metabolisms

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Abstract Despite its high capacity to take up nitrate from soil, winter rapeseed (*Brassica napus*) is characterized by a low N recovery in seeds. Thus, to maintain yield, rapeseed requires a high fertilization rate. Increasing nutrient use efficiency in rapeseed by addition of a biostimulant could help improve its agroenvironmental balance. The effects of marine brown seaweed *Ascophyllum nodosum* on plant growth have been well described physiologically. However, to our knowledge, no study has focused on transcriptomic analyses to determine metabolic targets of these extracts. A preliminary screening of different extracts revealed a significant effect of one of them (AZAL5) on rapeseed root (+102 %) and shoot (+23 %) growth. Microarray analysis was then used on AZAL5-treated or nontreated plants to characterize changes in gene expression that were further supported by physiological evidence. Stimulation of nitrogen uptake (+21 and +115 % in shoots and roots, respectively) and assimilation was increased in a similar manner to growth, whereas sulfate content (+63 and +133 % in shoots and roots, respectively) was more strongly stimulated leading to sulfate accumulation. Among the identified genes whose expression was affected by AZAL5, *MinE*, a plastid division regulator, was the most strongly affected. Its effect was supported by microscopic analysis showing an enhancement of chloroplast number per

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cell and starch content but without a significant difference in net photosynthetic rate. In conclusion, it is suggested that AZAL5, which promotes plant growth and nutrient uptake, could be used as a supplementary tool to improve rapeseed agroenvironmental balance.

Keywords *Brassica napus* · Seaweed extract · *Ascophyllum nodosum* · Microarray analysis · Growth promotion · Nutrient uptake · Chloroplast

Introduction

Any improvement in agricultural practices that increases plant nutrient capture efficiency should reduce the negative environmental impact of agriculture and increase crop production and sustainability in reduced input systems. Thus, many approaches have been studied to increase nutrient capture and yield, such as genetic selection, allele selection, selection of domestication genes, gene and genome duplication, new genotype creation, and quantitative trait loci (QTLs) (for review, see Vaughan and others 2007). Better knowledge of plant nutrient uptake and assimilation could also yield better efficiency from crop fertilization. Recent studies have focused principally on potentially polluting nutrients such as phosphorus (P) and nitrogen (N) (Agostini and others 2010; Borda and others 2011). New strategies such as the use of biological molecules that act as biostimulants have been evaluated. As defined by Zhang and Schmidt (1997), biostimulants correspond to "materials, other than fertilizers, that promote plant growth when they are applied in small quantities." In addition, Zhang and Schmidt (1997) considered biostimulants as "metabolic enhancers" that can be used to increase the effectiveness of conventional mineral fertilizers (Craigie 2011). Among these biostimulants, bioactive substances extracted from seaweeds are the most studied.

Since antiquity, seaweeds have been applied directly to soil as manure and as soil-conditioning agents (Blunden and Gordon 1986; Metting and others 1988; Temple and Bomke 1988; Hong and others 2007). The observed benefits of seaweed to the growth, health, and yield of crop plants have been attributed to (1) the supply of essential nutrients provided by degradation of organic matter and (2) the improvement of soil texture with an increase of water-holding capacity. However, the use of marine seaweed to fertilize crops has required development of preparation methods to facilitate transport over long distances. For example, a method for compressing seaweed or marine plants into compact transportable form was published more than 150 years ago (Gardissal 1857). More recently, new techniques to obtain seaweed extract (seaweed liquefaction) instead of gross seaweed, such as alkaline extraction (Milton 1952) or low-temperature milling (Hervé and Roullier 1977), have been used. These liquid seaweed extracts therefore are used more frequently as they facilitate transport and application to soil or as a foliar spray. Further studies of these liquid extracts have been performed to understand their potentialities and modes of action.

Studies on the effects of seaweed extracts have shown accelerated development cycles, that is, earlier germination, flowering, and fructification in treated grapevine and strawberry (Sivasankari and others 2006; Roussos and others 2009). Other authors have related an increase in total dry weight (DW) and, more specifically, the proliferation of secondary root systems in response to foliar spraying with seaweed extract on *Arabidopsis thaliana* (Rayorath and others 2008), grapevine (Mugnai and others 2008), and strawberry (Roussos and others 2009; Spinelli and others 2010). Enhancement of leaf chlorophyll content has also been reported in grapevine (Sivasankari and others 2006; Mancuso and others 2006) and strawberry (Spinelli and others 2010). This improved development and increase in crop growth led to an increase in yield, quality, and size of harvested products for grapevine, strawberry, soybean, tomato, and maize treated by foliar spraying of seaweed extract (Crouch and Van Staden 1992; Sivasankari and others 2006; Khan and others 2009; Rathore and others 2009; Roussos and others 2009). All these authors suggest that phytohormones contained in the seaweed extract, such as auxins or cytokinins, are probably responsible for the accelerated and improved development of plants. In this way, Khan and others (2011) demonstrate that liquid culture or foliar spraying with an *A. nodosum* extract induced cytokinin activity in *A. thaliana* leaves and roots.

Focusing more on plant metabolism, Mancuso and others (2006) showed that application of seaweed extract on grapevine increased the leaf accumulation of N, P, K, Mg, and Zn, with a particular increase in K^+ and Ca^{2+} influx into stomatal cells. Rathore and others (2009) also showed an accumulation of N, P, K, and S in the seeds of treated soybean. Durand and others (2003), focusing on N metabolism, reported that application of algae extract on *A. thaliana* (by foliar spraying or addition in the growth medium) enhanced nitrate content and nitrate reductase (NR) activity in leaves. In the case of increased yield and DW, the authors suggested that the effect of seaweed on nutrient metabolism may be due to the phytohormone content in the extracts. Therefore, the beneficial effect of seaweed extract application to plants seems to be the result of many components (phytohormones, betaines, polymers, nutrients) that may work synergistically. However, only a few studies have focused on metabolic targets in the interaction of seaweed extracts and plants (Durand and others 2003).

Winter oilseed rape (WOSR, *Brassica napus* L.) is an important agricultural crop cultivated for its oil, which can

be used as an edible product or for industrial application (for example animal nutrition, cosmetics, diester production). WOSR can be used as a catch crop to reduce N leaching during the autumn–winter period because of its high capacity to take up nitrate from soil (Laîne and others 1993). However, oilseed rape shows a low nitrogen use efficiency (NUE, defined by the ratio of seed N content to total plant N content), especially due to the default in leaf N mobilization (Etienne and others 2007; Desclos and others 2008) during the vegetative stage. As a consequence, N remaining in fallen leaves is a loss for dry matter production but also increases the risk of nitrate leaching following the mineralization of leaf organic N. Indeed, the fall of WOSR leaves with high N content (up to 2 % of the dry matter) (Malagoli and others 2005) leads to a return of N to the soil that can reach 100 kg N ha^{-1} (Dejoux and others 2000). Due to this low NUE, oilseed rape requires excessive N fertilization to maintain high N content of harvested tissues. Whatever the rate of N fertilization, the oilseed rape N harvest index (NHI) is low (approximately 70 %) (Dreccer and others 2000) compared with cereals. This has led to a defective agroenvironmental balance that might be improved by triggering an increased NUE (especially N) in oilseed rape by the addition of biostimulants. Thus, the aim of the present study was to identify new seaweed extracts that could increase the mineral nutrition and growth of WOSR so as to reduce the dose of fertilizer currently used.

To better understand the effects of an *A. nodosum* extract, named AZAL5, on WOSR growth, a fine characterization (elementary and hormonal composition) of this seaweed extract has been performed and coupled with transcriptomic (microarray), physiological, biochemical, and light and electron microscopy analyses. This approach allowed the identification of specific genes or groups of genes that were up- or down-regulated when WOSR was treated with AZAL5.

Materials and Methods

Growth Conditions and Experimental Design

Seeds of *B. napus* var. Capitol were surface-sterilized by exposure to 80 % ethanol for 30 s followed by 20 % sodium hypochlorite for 10 min. After ten washes with deionized water, seeds were germinated on perlite over deionized water for 2 days in the dark and 1 week in the light in a greenhouse. Just after first leaf emergence, seedlings were transferred to a 20 L plastic tank containing nutrient solution with the following composition: KNO_3 1 mM, $CaCl_2$ 1.25 mM, KCl 250 mM, KH_2PO_4 0.25 mM, $MgSO_4$ 0.5 mM, EDTA-2NaFe 0.2 mM, H_3BO_3 14 μM, $MnSO_4$ 5 μM, $ZnSO_4$ 3 μM, $CuSO_4$ 0.7 μM, $(NH_4)_6Mo_7O_{24}$ 0.7 μM, and $CoCl_2$ 0.1 μM. This nutrient solution was renewed every 2 days. Plants were grown under greenhouse conditions, with a thermo period of 20/17 °C day/night and a photoperiod of 16 h. Natural light was supplemented with high-pressure sodium lamps (Philips, MASTER Green Power T400W) supplying an average photosynthetically active radiation of 280 μmol photons m^{-2} s^{-1} at canopy height. Plants were grown for 1 week before treatment with AZAL5 seaweed extract.

Seaweed Extract Characterization (AZAL5)

Extraction and Composition of A. nodosum Extract AZAL5

The fresh algae harvested on Brehat Island shores in October 2008 was washed, shredded, and added to water. The solution was acidified with concentrated 95 % sulfuric acid to pH 3. The mixture was homogenized to microrupture the algal cells then centrifuged and filtered. The amount of biologically active extracted compounds varies by season and also by environmental conditions. The final solution was then concentrated as described by Briand (1998) to obtain dry AZAL5 extract. Before use, the dry extract was dissolved in water at a concentration of 67 gL^{-1}.

Analysis of the Concentration of IAA, ABA, and Cytokinins in AZAL5

The general method is extensively described in Aguirre and others (2009) and Mora and others (2010).

Plant Treatment

After 1 week of growth, plants were separated into two sets: (1) control plants (control) were grown in the nutrient solution described above with ^{15}N labeling (3 % atom excess), (2) treated plants (AZAL5) were grown in the same labeled solution supplied with 0.1 % (v/v) of AZAL5 seaweed extract. Nutrient solutions were renewed every 2 days. Both sets of plants (control and AZAL5) were grown for 30 days and time courses in the experiment were expressed in days after the beginning of treatment (Day 0, addition of AZAL5 to the nutrient solution).

For each condition (control and treatment with AZAL5) and each duration of treatment (1, 3, and 30 days), ten plants (that is, ten plants pooled in three replicates) were harvested and separated for shoot and root samples. The roots and shoots were frozen in liquid nitrogen and stored at −80 °C for further analysis. An aliquot of each tissue was weighed and dried (60 °C) in a drying oven for DW determination and ground to fine powder for mineral and

ion analysis. Likewise, at each time of harvest, a fresh aliquot of shoots and roots was used for real-time in vivo NR activity (NR_{act}).

Determinations of Chlorophyll Concentration and Net Photosynthetic Rates

During the experiment, determinations of chlorophyll concentration and net photosynthetic rates were monitored at 1 and 3 days after AZAL5 application and then weekly until the end of treatment (that is, 30 days). Determination of relative chlorophyll concentration was performed using a nondestructive method: SPAD (soil plant analysis development) chlorophyll meter (SPAD-502 model, Minolta, Tokyo, Japan). The determination was carried out on ten measures per leaf and on five leaves per replicate. The net photosynthetic rate was measured using a LI-6400 (LICOR, Lincoln, NE, USA) at 23 °C on leaves under ambient CO_2 atmospheric concentration ($\sim$400 ppm) and at 1,000 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by a LED light. Temperature and PPF parameters were previously validated as the optimal values for photosynthesis analysis in leaves of oilseed rape.

Mineral and Ion Quantification

Total N and ^{15}N Analysis

An aliquot of each plant organ (shoots or roots) was placed in thin capsules for isotopic analysis of between 60 and 80 µg N. The total N amount and ^{15}N excess in plant samples were determined with a continuous flow isotope mass spectrometer (Isoprime, GV Instrument, Manchester, UK) linked to a C/N/S analyzer (EA3000, Euro Vector, Milan, Italy):

Total N (N_{tot}) content in a tissue i at a given time was calculated as

$$N_{tot_i} = (\%N_i \times DW_i)/100$$

The natural ^{15}N abundance (0.36636 ± 0.0004 %) of atmospheric N_2 was used as a reference for ^{15}N analysis. Nitrogen derived from current N uptake (N_{upt_i}) in a given organ, at a given time, was calculated as

$$N_{upt_i} = (N_{tot_i} \times E_i)/E_s$$

where E_i (%) is the atom ^{15}N excess in a given tissue (shoots or roots) and E_s is the nutrient solution atom ^{15}N excess (3 %).

Total S Analysis

The total S amount in shoots and roots was measured by ICP-OES (Thermo Elemental Co. Iris Intrepid II XDL) with prior microwave acid sample digestion (8 ml of concentrated HNO_3 and 2 ml of H_2O_2 for 0.5 g DW) as previously described by Mora and others (2010).

Nitrate and Sulfate Analysis

Nitrate and sulfate were extracted and analyzed as previously described (Abdallah and others 2011) from 30 mg of DW (shoots or roots) in 1.5 ml of 50 % ethanol solution at 40 °C for 1 h. After centrifugation (10,000×g for 20 min), the supernatant (called S1) was recovered and 1.5 ml of 50 % ethanol was added to the pellet. After a second incubation (40 °C for 1 h) and centrifugation (10,000×g for 20 min), the remaining supernatant was taken up and added to the previous supernatant (S1). All these operations (that is, incubation and centrifugation) were repeated twice but now with 1.5 ml of ultrapure water and incubation at 95 °C. All supernatants were finally pooled then dried under vacuum for 16 h without heating (Concentrator Evaporator RC 10.22 Jouan, Saint Herblain, France). The dry residues containing both nitrate and sulfate were solubilized in 1 ml of ultrapure water. Thereafter, nitrate and sulfate concentrations in each tissue were determined by using ion chromatography (HPLC, ACS3000, Dionex Corp., Sunnyvale, CA, USA) with a conductivity detector. The eluent solution consisted of 1.8 mM Na_2CO_3 and 1.7 mM Na_2HCO_3 and was pumped isocratically over an AS17 guard column.

In vivo NR_{act} in Plants

The NR_{act} was determined in each replicate using an in vivo assay adapted from Bungard and others (1999). Shoot or root tissue (0.1 g FW) was vacuum infiltrated for 3 times 30 s with 10 ml of phosphate buffer (pH 7.5) containing 1 % (v/v) propanol and 1 M KNO_3. After vacuum infiltration, buffer solutions containing plant material were separated in two sets. One part was incubated in a shaking water bath at 30 °C for 15 min in darkness and then boiled to stop any enzymatic activity. The other part was boiled immediately after vacuum infiltration for initial nitrite concentration determination. The colorimetric reaction was performed with 1 ml of plant extract, 1 ml of 0.3 % (w/v) sulfanilic acid in 30 % acetic acid, and 1 ml of 0.05 % (w/v) α-naphthylamine in 30 % acetic acid. The amount of nitrite (NO_2^-) formed in each buffer solution was measured spectrophotometrically (UV-1601, UV–Visible spectrophotometer, Shimadzu, Champs-sur-Marne, France) at 540 nm. Thereafter, NR_{act} was calculated as µmol NO_3^- reduced per g FW and per hour.

RNA Extraction

Total RNA was extracted from 200 mg of root and shoot FW. Frozen samples were ground to a powder with a pestle

in a mortar containing liquid nitrogen. The resulting powder was suspended in 750 µL of extraction buffer [0.1 M Tris, 0.1 M LiCl, 0.01 M EDTA, 1 % SDS (w/v), pH 8] and 750 µL of hot phenol (80 °C, pH 4). This mixture was vortexed for 30 s, and after addition of 750 µL of chloroform/isoamyl alcohol (24:1), the homogenate was centrifuged at 15,000×*g* (5 min, 4 °C). The supernatant was transferred into 4 M LiCl solution (w/v) and incubated overnight at 4 °C. After centrifugation (15,000×*g* for 30 min at 4 °C), the pellet was suspended in 100 µL of sterile water. RNA was then purified using the RNeasy mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). Quantification of total RNA was performed by spectrophotometry at 260 nm (BioPhotometer, Eppendorf, Hamburg, Germany) before reverse transcription (RT) and real-time quantitative PCR (q-PCR) analysis.

Reverse Transcription and q-PCR Analysis

For RT, 1 µg of total RNA was converted to cDNA with an iScript cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Quantitative PCR amplifications were performed using specific primers for each housekeeping gene (EF1-α [forward 5′-gcctggtatggttgtgacct-3′ and reverse 5′-gaagtta gcagcacccttgg-3′] and RNA 18S [forward 5′-cggataaccgtag taattctag-3′ and reverse 5′-gtactcattccaattaccagac-3′]) and target gene: BnNRT1.1 forward 5′-tggtggaataggcggctcgag ttg-3′ and reverse 5′-gtatacgttttgggtcattgccat-3′, BnNRT2.1 forward 5′-atggtaacggaagtgccttg-3′ and reverse 5′-tgattcg agctgtgtgaagc-3′, BnSultr1.1 forward 5′-agatattgcgatcgga ccag-3′ and reverse 5′-gaaaacgccagcaaagaaag-3′, BnSult r1.2 forward 5′-ggtgtagttcgctggaatggt-3′ and reverse 5′-aa cggagtgaggaagagcaa-3′, BnSultr4.1 forward 5′-gaccagaccc gttaaggtca-3′ and reverse 5′-ttggaatccatgtgaagcaa-3′, BnSultr4.2 forward 5′-agcaagatcagggattgtgg-3′ and reverse 5′-tgcaacatttgtgggtgtct-3′.q-PCRs were performed with 4 µL of 100× diluted cDNA, 500 nM of primers, and 1× SYBR® Green PCR Master Mix (Bio-Rad) in a Chromo-Four System (Bio-Rad). For each pair of primers, a threshold value and PCR efficiency were determined using a cDNA preparation diluted more than tenfold. For all pairs of primers, PCR efficiency was approximately 100 %. The specificity of PCR amplification was examined by monitoring the presence of the single peak in the melting curves after q-PCRs and by sequencing the q-PCR product to confirm that the correct amplicons were produced from each pair of primers (Biofidal). For each sample, the subsequent q-PCRs were performed in triplicate. The relative expression of the genes in each sample was compared with the control sample (corresponding to untreated plants at the same time of harvest) and was determined with the $\Delta\Delta C_t$ method using the following equation (Livak and Schmittgen 2001):

$$\text{Relative expression} = 2^{-[\Delta C_t \text{treated} - \Delta C_t \text{control}]},$$

with

$$\Delta C_t = C_{t\ \text{targetgene}} - [\sqrt{(C_{t\ \text{EF1}-\alpha} \times C_{t\ 18S})}],$$

where C_t refers to the threshold cycle determined for each gene in the exponential phase of PCR amplification and $[\sqrt{(C_{t\ \text{EF1-}\alpha} \times C_{t\ 18S})}]$ corresponds to the geometric average of C_t of the reference genes. Using this analysis method, relative expression of the different genes in the control sample of the experiment was equal to 1 (Livak and Schmittgen 2001), and the relative expression of other treatments was then compared with the control on this basis.

Microarray Analysis

Briefly, each test sample was hybridized on a *B. napus* Gene Expression Microarray 4 × 44 K (Agilent Technologies, Palo Alto, CA, USA) using a two-color microarray-based gene expression protocol. In this procedure, controls and samples treated with AZAL5 were compared and respectively labeled with cyanine 3 and cyanine 5. For each plant tissue (shoots and roots) and each time of harvest (1, 3, and 30 days), the same control was used for the different hybridizations. After the labeling step, cRNA sample sizes ranged from 50 to 3,000 nucleotides. Thus, fragmentation was required to take away secondary structures (specific buffer at 60 °C for 30 min) enabling a cRNA length of between 50 and 200 nucleotides to be obtained and optimal hybridization with Agilent 60-mer oligonucleotide microarrays to be carried out. Thereafter, hybridizations were performed at 65 °C for 17 h.

Scanning of microarrays was performed using the Agilent scanner with default parameters for 4 × 44 K formats. Data were extracted using Feature Extraction 10.1 software (Agilent Technologies).

Fluorescence Confocal Microscopy and Transmission Electron Microscopy (TEM)

For microscopy experiments, the choice of the leaf observed is important. After 1 and 3 days of contact with AZAL5, observations were made on young plants with four leaves. We then chose to make observations on the last fully extended leaf: leaf No. 3. After 30 days of treatment, we chose to make the observations on a leaf in the center of the plant. The first leaves that had emerged were nearly senescent and young leaves just emerged were not representative of the whole plant. Thus, among the eight leaves that had emerged by the end of culture, we then chose to focus our observations on leaf No. 5.

A square of rapeseed leaf of each replicate of each treatment (control and AZAL5-treated plant) and each time of harvest (1, 3, and 30 days) was embedded in LMPT (low melting point) agar 5 % in phosphate buffer at 40 °C. After cooling, 50-µm-thick slices were cut with a vibratome (Microm HM650 V). Half of these slices were observed directly with confocal microscopy (Olympus FV1000) with laser excitation of autofluorescence at 458 nm and emission at 650–700 nm. The remaining slices were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) from 1 h to several days at 4 °C. The sections were rinsed in 0.1 M phosphate buffer (pH 7.4) three times, post-fixed for 1.5 h with 1 % osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), and then rinsed in phosphate buffer three times. The cells were then dehydrated in progressive baths of ethanol (70–100 %) and propylene oxide 100 %, embedded in Aradite 502 resin, and polymerized for 48 h at 60 °C. Ultrathin sections of 80 nm were cut and contrasted with uranyl acetate and lead citrate. The sections were observed with a JEOL 1011 transmission electron microscope, and images were taken with a MegaView 3 camera and analysis five software.

Table 1 Elemental nutrient composition of seaweed extract AZAL5 dissolved in water at a concentration of 67 g DW L^{-1}

Element	Concentration (ppm)	Element	Concentration (%)
Ca	572	C	1.79
Cu	6	H	9.89
Fe	20	N	0
K	4,442	O	87.3[a]
Mg	616		
Na	2,078		
P	78		
S	1782		
Si	18		
Zn	1.2		

[a] Determination by difference

Data and Statistical Analyses

Regarding growth, nitrate uptake, SPAD, and IRMS analysis, experiments were conducted with ten replicates. In the case of ICP and DIONEX analysis, experiments were conducted with six replicates. For microscopy, q-PCR, and microarray experiments, three replicates were used for each sample. All data were analyzed for significant differences by Student's *t*-test and marked by asterisks ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

Microarray

For each experiment (each time and each tissue), probes with a [Marginal] flag and at least one channel above the background for the three biological replicates were retained. A *t* test was applied on each filtered gene list with the following parameters: (1) *t* test against zero, (2) Benjamini-Hochberg correction, and (3) $p < 0.05$. A fold change was also made on the filtered list, but no statistical test evaluates the significance of these lists.

Results

Seaweed Extract (AZAL5) Characterization

Except for C, H, and O, which are the main components (1.7, 9.8, and 87.3 % respectively), AZAL5 extract principally contains Ca, K, Mg, Na and S. Surprisingly, this seaweed extract did not contain significant N (Table 1). Hormone analysis (Table 2) shows only very small amounts of auxin, abscisic acid, and cytokinins, especially iP (16.11 pmol g^{-1}) and iPR (0.46 pmol g^{-1}).

Growth Analysis

The addition of 0.1 % (v/v) of AZAL5 seaweed extract in the root growth medium did not affect significantly the total DW of rapeseed after 1 or 3 days of treatment (Fig. 1a). The total DW of control plants was 0.30 ± 0.03 and 0.57 ± 0.02 g

Table 2 Hormonal composition of seaweed extract AZAL5

Phytohormone	IAA	ABA	Z	DHZ	tZR	cZR	DHZR
Content	7.53	17.63	ND	ND	ND	ND	ND
Phytohormone	iP	iPR	BAR	mT	mTR	oT	oTR
Content	16.11	0.46	ND	ND	ND	ND	ND

Contents are expressed in pmol g^{-1}. Phytohormones measured were auxin (IAA), abscisic acid (ABA), and cytokinins zeatin (Z), dihydrozeatin (DHZ), *trans*-zeatin (tZR), *cis*-zeatin (cZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), isopentenyladenosine (iPR), benzyladenine riboside (BAR), meta-topolin (mT), meta-topolin riboside (mTR), ortho topolin (oT), and ortho-topolin riboside (oTR)

ND not detected

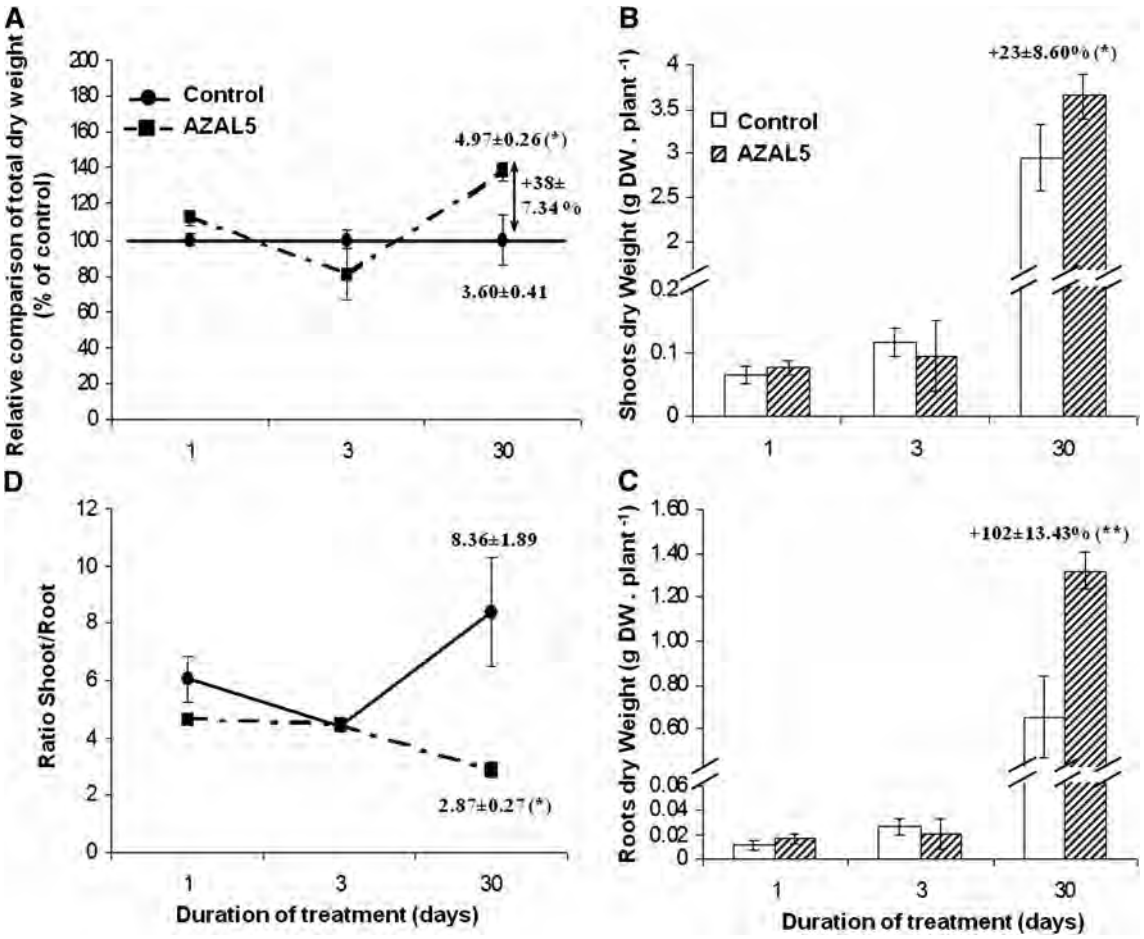

Fig. 1 Effect of seaweed extract on rapeseed dry weight (DW) after 1, 3, or 30 days of treatment. **a** Relative comparison of total dry weight of treated plants (*squares* and *dotted line*) to that of control (*circles* and *solid line*), in the percentage of control plants. Values near the points are the total DW expressed in g. **b** Shoot DW of control (*white histogram*) and treated plants (*hatched histogram*), expressed in g plant^{-1}. **c** Root DW of control (*white histogram*) and treated plants (*hatched histogram*), expressed in g plant^{-1}. **d** Shoot/root ratio of control (*circles* and *solid line*) and treated plants (*squares* and *dotted line*). Values are means and *vertical bars* indicate ±standard deviation for $n = 10$ when exceeding the symbol. Significant differences at $^{*}p < 0.05$ and $^{**}p < 0.01$

plant^{-1} after 1 and 3 days, respectively, compared with 0.37 ± 0.01 and 0.57 ± 0.02 g plant^{-1} for treated plants at the same time of harvest. After 30 days, plants treated with AZAL5 showed a significant increase in total DW (+38 ± 7.34 %) compared to control plants (3.60 ± 0.41 g plant^{-1} for control versus 4.97 ± 0.26 g plant^{-1} for treated plant). As shown in Fig. 1b, c, this increase in total DW could be explained by a significantly increased shoot DW (+23 ± 8.60 % compared to control, Fig. 1b), and more particularly by a high increase in root DW (+102 ± 13.43 % compared to control, Fig. 1c) after 30 days of treatment withAZAL5. At day 30, this higher DW in roots than in shoots (+23 ± 8.60 % DW) resulted in a lower shoot/root ratio for treated plants compared to control plants (8.36 ± 1.89 and 2.87 ± 0.27 in control and treated plants, respectively) (Fig. 1d).

When the nutrient composition of the solutions was analyzed (Table 3), it appears that AZAL5's contribution to the mineral supply of Hoagland solution was negligible (from +0.25 % for Fe to +12 % for Cu, with a notable contribution of Na: +25 %). Furthermore, regarding plant nutrient uptake by both control and treated plants (Table 3), whichever nutrient was considered, the fraction taken up by the plants was always lower than 15 % of the total nutrient supply. Thus, even in the controls, plants were not exposed to any limiting conditions and the increase in DW for treated rapeseed (Fig. 1) did not result from the amelioration of any kind of starvation.

Microarray Data

A total of 31,561 genes were analyzed on the microarray, creating an expression profile for each plant tissue and each time of treatment. Hierarchical clustering of these expression profiles (Fig. 2) shows that the three replicate profiles of each date × treatment were very close. The analysis of

Table 3 Nutrient composition of Hoagland solution and AZAL5 contribution to the nutrient supply

Element	Content in Hoagland solution(mg)	AZAL5 contribution (mg)	Control plant uptake (%)	AZAL5 plant uptake (%)
Ca	10,000	114.40	7.65	12.56
Cu	10	1.20	3.60	8.04
Fe	1,600	4.00	1.16	1.68
K	12,200	888.40	9.28	14.52
Mg	2,500	123.20	5.47	9.06
N	3,498	0	39.62	65.35
Na	1,606	415.60	0.43	0.56
P	2,860	15.60	6.80	10.14
S	4,440	356.40	7.82	12.87
Si	154	3.60	2.57	5.48
Zn	48	0.24	3.38	4.48

Total uptake for each nutrient over 30 days is shown resulting from the nutrient solution (control) or from the nutrient solution and AZAL5 supply (treated)

significantly and differentially expressed genes between control and treated plants was undertaken using a minimal fold change of expression of 5 ($p < 0.05$). Using this filter, no differentially expressed genes were found in shoots and roots after 1 day of treatment with AZAL5. After 3 days of treatment with AZAL5, microarray analysis revealed that 724 and 298 genes were differentially expressed in shoots and roots, respectively (Fig. 3a). After 30 days of AZAL5 treatment (Fig. 3b), it was shown that 612 and 439 genes were differentially expressed in shoots and roots of plants, respectively.

All differentially expressed genes have been classified in metabolic pathways according to DFCI annotations (http://compbio.dfci.harvard.edu, Fig. 3). From this global classification, at first sight it appears that about 60 % of differentially expressed genes did not have a known function (supplemental data, Supplementary Tables 1 and 2) resulting from the lack of complete sequencing of the *B. napus* genome. However, the DFCI classification revealed that several metabolic pathways were affected by AZAL5 treatment (Fig. 3a, b). Among these, some metabolic pathways (such as fatty acids, phytohormones, senescence, plant development, and ion transport) were represented in low numbers among the genes that were differentially expressed. In contrast, four metabolic pathways were more specifically affected in shoots and roots by the treatment (Fig. 3a, b): general cell metabolism (10.3 % of the total differentially expressed genes on average), carbon metabolism and photosynthesis (7.1 % on average), stress responses (6.7 % on average), and nitrogen and sulfur metabolism (5.9 % on average). Thus, according to our initial goal, which was to target an improvement in nutrient use efficiencies (such as N and S) of WOSR following seaweed treatment, this study focused on N, S, and C metabolism (especially photosynthesis).

In shoots (Table 4), 62 % of genes involved in photosynthetic pathways were downregulated, most of them being labeled "chloroplast precursor," such as the gene encoding protochlorophyllide reductase B, an enzyme involved in chlorophyll biosynthesis (Ougham and others 2001), or the gene encoding a plastid division regulator, *AtMinE* (Itoh and others 2001), whose expression was reduced more than 50-fold after 3 days. Other downregulated genes encoded proteins involved in chlorophyll degradation, such as genes encoding chlorophyllase-1 or stay green protein (Sgr). This microarray analysis also revealed that 38 % of genes involved in photosynthesis were upregulated, such as genes encoding ferredoxin and photosystem proteins, that is, proteins implicated in the electron transport chain.

Concerning genes involved in N metabolism, Table 4 reveals that 60 % of genes were upregulated. Among these upregulated genes were found genes encoding enzymes involved in nitrate assimilation, such as NR (+32.908- and +8.385-fold at day 3 and 30, respectively), or genes involved in amino acid metabolism. Among the genes that

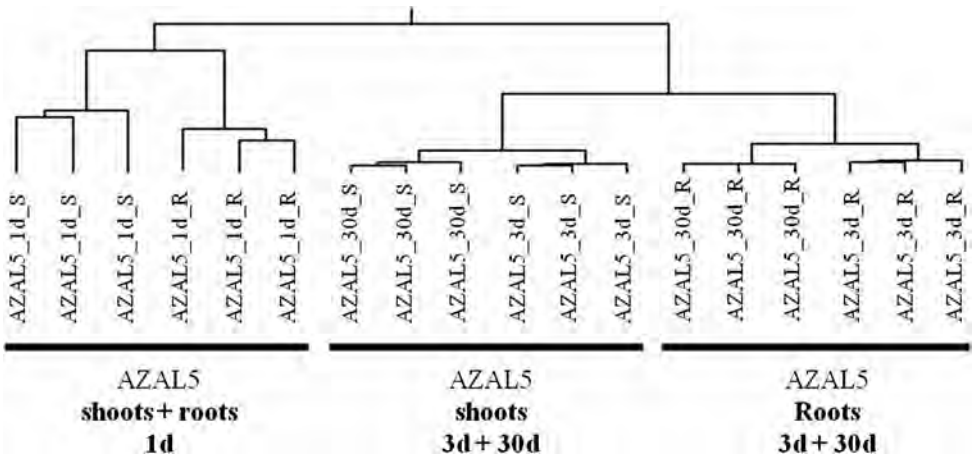

Fig. 2 Hierarchical clustering of microarray profiles. *Profiles with the same name* indicate the three experimental replicates for each time [1 day (d), 3 days, and 30 days] of treatment with AZAL5 extract. *Bold bars* represent expression groups logically defined by the clustering

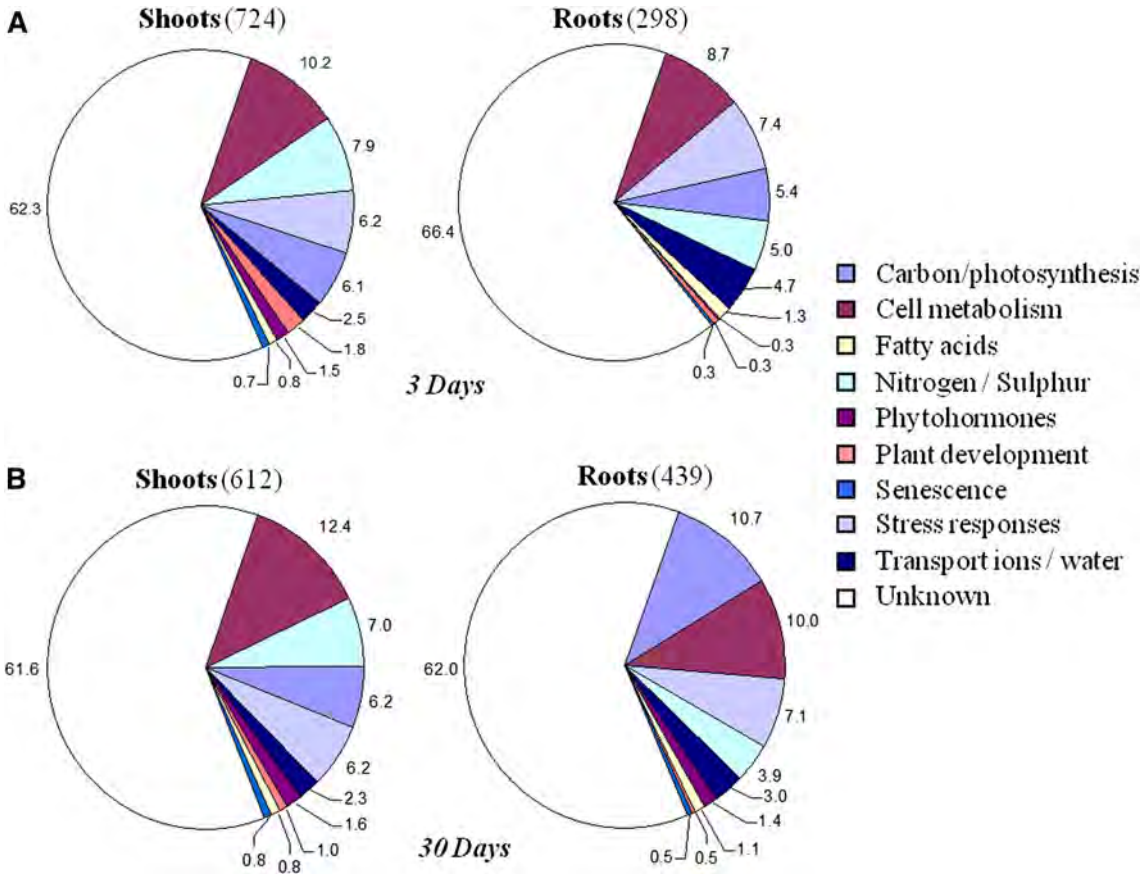

Fig. 3 Distribution among metabolic pathways (according to DFCI annotation) of genes differentially expressed in *Brassica napus* roots and shoots after 3 days (**a**) or 30 days (**b**) of treatment with AZAL5. *Numbers in parenthesis* indicate the total number of genes differentially expressed in each condition (each time of treatment and each part of the plant, $p < 0.05$). *Numbers near pie charts* indicate the percentage of differentially expressed genes implicated in each metabolic pathway

were downregulated, some proteases, and more specifically a senescence-associated cysteine protease, were strongly downregulated (Table 4) by the seaweed extract (−94.345- and −73.099-fold at day 3 and 30, respectively).

For the S metabolic pathway (Table 4), around 80 % of genes were upregulated. Some of these genes encoded proteins involved in sulfate uptake and assimilation, such as sulfate transporters, ATP sulfurylases, and serine acetyltransferases. Moreover, seaweed extract induced some genes encoding the Tau and Phi classes of glutathione-S-transferases, which are plant-specific multifunctional proteins that perform pivotal catalytic and non-catalytic functions in normal plant development and stress responses (for review, see Moons 2005). A low proportion of S metabolism-related genes were downregulated: only 20 and 7 % at 3 and 30 days, respectively.

Microarray analysis of roots (Table 5) revealed that unlike shoots, most of the genes were differentially expressed after only 30 days of treatment. In the photosynthetic pathway, most upregulated genes (79 %), such as those encoding photosystem I reaction center subunits or plastocyanin isoform, were labeled "Photosystem." In common with shoots, an upregulation of ferredoxin (ferrodoxin-2) and a strong downregulation of genes encoding the plastid division regulator AtMinE occurred at 3 and 30 days (−18.782- and −35.693-fold at day 3 and 30, respectively). Focusing on differentially expressed genes in roots related to N metabolism, there was a substantial downregulation of some genes encoding protease inhibitors at day 3 and 30 and a downregulation of some cysteine protease RD19 genes only at day 30. Focusing on S metabolism, sulfate transporter genes (high- and low-affinity sulfate transporters) were upregulated as previously observed in shoots (Table 4). Moreover, glutathione-S-transferases and serine acetyltransferase genes were downregulated and upregulated, respectively, and mostly at 3 and 30 days.

N Metabolism

The total N amount in treated plants (Fig. 4a) revealed that after 30 days, treatment with AZAL5 significantly affected N content in plants. Thus, at day 30, the N contents in

Table 4 Partial list of differentially expressed shoot genes involved in photosynthesis, nitrogen and sulfur metabolism after 3 or 30 days of treatment with AZAL5 extract

Pathway	Accession no.	Description	Shoots 3 days		Shoots 30 days	
			Corrected *p* value	Fold change	Corrected *p* value	Fold change
Photosynthesis	NP174327	At—carbonic anhydrase 1, chloroplast precursor	**0.007**	**5.666**	**0.012**	**5.507**
	AY433944	Br—early light-inducible protein (ELIP)	**0.007**	**8.790**	**0.009**	**6.905**
	CP002684	At—ferredoxin-2, chloroplast precursor	**0.009**	**6.184**	**0.011**	**7.917**
	DQ244819	At—ferredoxin-2, chloroplast precursor	**0.008**	**6.958**	**0.008**	**7.863**
	AJ312190	At—ferritin-3, chloroplast precursor	**0.007**	**6.814**	**0.010**	**7.299**
	NM123450	At—*S*-adenosyl-L-methionine-dependent uroporphyrinogen III methyltransferase	**0.007**	**6.706**	**0.009**	**5.207**
	NM104609	At—glutamyl-tRNA reductase 1, chloroplast precursor	**0.013**	**6.074**	–	–
	AC189413	Gt—photosystem II reaction center protein Z	**0.010**	**5.809**	–	–
	CP002688	At—pyridoxal biosynthesis protein PDX1.3	–	–	**0.012**	**5.032**
	NM100952	At—alpha-glucan water dikinase 1, chloroplast precursor	*0.007*	*−29.094*	*0.011*	*−19.076*
	AF458411	At—chaperone protein dnaJ 8, chloroplast precursor	*0.012*	*−5.118*	*0.009*	*−6.155*
	GR444715	At—chlorophyllase	*0.007*	*−5.914*	*0.015*	*−7.253*
	AJ635427	At—phosphoglucan, water dikinase, chloroplast precursor	*0.012*	*−11.756*	*0.025*	*−10.951*
	DQ118104	Bn—plastid division regulator MinE	*0.010*	*−51.566*	*0.022*	*−26.060*
	NM118879	At—protochlorophyllide reductase B, chloroplast precursor	*0.007*	*−6.188*	*0.009*	*−9.892*
	AY699948	Ca—stay green protein (sgr)	*0.016*	*−13.480*	*0.027*	*−6.204*
	NM129396	At—alanine–glyoxylate aminotransferase 2 homolog 2	*0.010*	*−11.735*	–	–
	NM120081	At—glucose-1-phosphate adenylyltransferase large subunit 3	*0.008*	*−6.202*	–	–
	NM100952	At—alpha-glucan water dikinase 1, chloroplast precursor	–	–	*0.017*	*−6.916*
	AF458411	At—chaperone protein dnaJ 8, chloroplast precursor	–	–	*0.010*	*−5.452*
	AF337544	Bo—chlorophyllase 1	–	–	*0.017*	*−5.872*
	NM100115	At—long hypocotyl in far-red 1 (HFR1)	–	–	*0.013*	*−122.894*
	AB456972	Al—phytochrome B	–	–	*0.015*	*−18.605*
Nitrogen metabolism	NM100380	At—1-aminocyclopropane-1-carboxylate oxidase homolog 1	**0.008**	**6.195**	**0.008**	**5.635**
	NM121798	Mc—5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	**0.008**	**25.834**	**0.040**	**20.595**
	AY128334	At—anthranilate *N*-hydroxycinnamoyl/benzoyltransferase-like protein	**0.017**	**8.634**	**0.016**	**11.211**
	NP195197	Bn—arginine decarboxylase 2	**0.005**	**15.447**	**0.020**	**6.446**
	ES912832	Bn—asparaginase	**0.010**	**5.832**	**0.047**	**7.636**
	FJ830448	Bn—epithiospecifier modifier (ESM)	**0.012**	**9.538**	**0.016**	**11.750**
	NM127123	At—ferredoxin-nitrite reductase NiR1	**0.025**	**12.794**	**0.011**	**12.576**
	NP181221	Bn—gamma tonoplast intrinsic protein	**0.007**	**5.970**	**0.011**	**6.465**

Table 4 continued

Pathway	Accession no.	Description	Shoots 3 days		Shoots 30 days	
			Corrected *p* value	Fold change	Corrected *p* value	Fold change
	NM111956	At—GDSL-motif lipase/hydrolase-like protein	**0.007**	**5.805**	**0.014**	**5.756**
	XM002872757	Al—*N*-hydroxycinnamoyl/benzoyltransferase-like protein	**0.014**	**14.758**	**0.019**	**7.090**
	D38220	Bn—nitrate reductase	**0.013**	**32.908**	**0.010**	**8.385**
	NM179668	At—tyrosine decarboxylase 1	**0.024**	**18.593**	**0.014**	**19.571**
	NM127071	At—3-ketoacyl-CoA synthase 8	**0.014**	**7.361**	–	–
	NM118831	At—aconitate hydratase 2 (ACO2)	**0.013**	**7.801**	–	–
	AY337608	Bj—arginine decarboxylase	**0.032**	**8.508**	–	–
	NM180941	At—asparagine synthetase (ASN2)	**0.007**	**9.573**	-	–
	DY022560	Bn—aspartate kinase	**0.007**	**6.059**	–	–
	DQ485132	Bn—cinnamate 4-hydroxylase isoform 2 (C4H)	**0.008**	**5.354**	–	–
	NM113299	At—dihydroxy-acid dehydratase	**0.008**	**5.851**	–	–
	NM114620	At—malate dehydrogenase chloroplast precusor	**0.007**	**5.009**	–	–
	NM111869	At—phenylalanine ammonia-lyase 4	**0.008**	**5.339**	–	–
	DQ341308	Bn—phenylalanine ammonia-lyase (PAL1-1)	**0.007**	**5.192**	–	–
	CN729283	At—cysteine proteinase	–	–	**0.019**	**5.027**
	NM121480	At—L-aspartate oxidase-like protein	–	–	**0.011**	**9.487**
	NM202733	At—strictosidine synthase	–	–	**0.010**	**6.511**
	NP194437	At—tryptophan synthase beta subunit (TSB2)	–	–	**0.010**	**5.202**
	NP566700	At—4-aminobutyrate transaminase	*0.007*	*−6.688*	*0.010*	*−5.147*
	NP001078093	At—adenosylmethionine decarboxylase	*0.047*	*−7.921*	*0.012*	*−6.539*
	NM120900	At—agmatine deiminase	*0.007*	*−8.094*	*0.010*	*−6.261*
	X77260	Bn—aspartic protease	*0.008*	*−6.701*	*0.007*	*−7.894*
	AM501059	St—aspartic protease-like	*0.009*	*−6.558*	*0.008*	*−5.851*
	BT000269	At—branched-chain alpha keto-acid dehydrogenase E1 alpha subunit	*0.012*	*−10.398*	*0.022*	*−10.971*
	AF454959	At—cysteine proteinase RD19A	*0.007*	*−13.209*	*0.008*	*−10.448*
	XP002883348	Al—gamma-aminobutyrate transaminase subunit precursor	*0.007*	*−6.378*	*0.009*	*−5.160*
	AK317177	At—glyoxalase I protein family	*0.009*	*−24.760*	*0.028*	*−12.288*
	AF089848	Bn—senescence-specific cysteine protease	*0.008*	*−94.345*	*0.009*	*−73.099*
	NM100380	At—1-aminocyclopropane-1-carboxylate oxidase homolog 1	*0.021*	*−5.641*	–	–
	NM119749	At—aconitate hydratase 1 (ACO1)	*0.007*	*−5.670*	–	–
	EE567694	Bn—calmodulin	*0.008*	*−5.092*	–	–
	NM119466	At—coclaurine *N*-methyltransferase	*0.010*	*−6.678*	–	–
	U51119	Bc—cysteine proteinase inhibitor	*0.012*	*−5.257*	–	–
	NP188895	At—GCN5-related *N*-acetyltransferase protein family	*0.025*	*−6.925*	–	–
	NM129089	At—Glycine cleavage system H protein 1	*0.008*	*−6.671*	–	–
	NM127548	At—malic enzyme	*0.026*	*−5.671*	–	–
	NM117360	At—serine carboxypeptidase-like 20 precursor	*0.007*	*−5.194*	–	–

Table 4 continued

Pathway	Accession no.	Description	Shoots 3 days		Shoots 30 days	
			Corrected *p* value	Fold change	Corrected *p* value	Fold change
	EV093737	At—1-aminocyclopropane-1-carboxylate oxidase homolog 1	–	–	*0.037*	*−6.259*
	AF314811	Bn—delta 1-pyrroline-5-carboxylate synthetase A	–	–	*0.015*	*−6.784*
Sulfur metabolism	NM120157	At—adenylylsulfate kinase	**0.009**	**8.627**	**0.008**	**8.572**
	U68218	Bn—ATP sulfurylase (LSC680)	**0.008**	**12.058**	**0.009**	**8.099**
	EU346738	Bo—ATP sulphurylase 1 precursor (ATPS1)	**0.007**	**10.864**	**0.012**	**7.215**
	AJ223499	Bj—ATP sulfurylase precursor	**0.008**	**11.366**	**0.013**	**7.327**
	AY097392	Bj—glutathion *S*-transferase 1	**0.019**	**5.376**	**0.017**	**7.819**
	DQ091257	Bn—putative low-affinity sulfate transporter	**0.007**	**10.448**	**0.013**	**9.670**
NM111114	At—*S*-adenosylmethionine decarboxylase	**0.007**	**8.569**	**0.012**	**6.419**	
HM013966	Bn—*S*-adenosylmethionine decarboxylase	**0.009**	**11.527**	**0.016**	**6.223**	
U63734	At—*S*-adenosyl-L-methionine-dependent uroporphyrinogen III	**0.007**	**8.389**	**0.010**	**6.318**	
AJ416461	Bn—sulfate transporter (stp1 gene)	**0.008**	**8.363**	**0.015**	**5.767**	
NM104662	At—glutathione *S*-transferase TAU16 (GSTU16)	**0.009**	**5.202**	–	–	
NP187918	At—serine acetyltransferase (SERAT2.2)	**0.007**	**6.843**	–	–	
NM118383	At—adenine phosphoribosyltransferase-like protein	–	–	**0.012**	**8.499**	
EV118979	At—glutathione *S*-transferase	–	–	**0.017**	**7.819**	
AF304430	Bn—thiohydroximate *S*-glucosyltransferase	–	–	**0.008**	**5.159**	
DQ059298	Bo—epithiospecifier protein	*0.008*	*−54.721*	*0.008*	*−30.526*	
NM118319	At—autophagy-related protein 8A (APG8A)	*0.017*	*−6.414*	–	–	
FJ357244	Th—glutathione peroxidase 6 (GPX6)	*0.008*	*−5.287*	–	–	

The first two letters of the gene description indicate the species: Al = *Arabidopsis lyrata*; At = *Arabidopsis thaliana*; Bc = *Brassica campestris*; Bj = *Brassica juncea*; Bn = *Brassica napus*; Bo = *Brassica oleracea*; Br = *Brassica rapa*; Bu = *Bauhinia ugulata*; Ca = *Capsicum annuum*; Cp = *Citrus paradisi*; Gt = *Guillardia theta*; Mc = *Mesembryanthemum crystallinum*; Nt = *Nicotiana tabacum*; St = *Solanum tuberum*; Th = *Thellungiella halophila*

Positive fold change indicates that the gene is specifically overexpressed in response to seaweed extract (*bold*); negative fold change indicates that the gene is specifically repressed in response to seaweed extract (*italic*)

– Indicate genes with expression levels that are not significantly different from control

p values are Bonferroni-corrected. Genes were considered as differentially expressed at $p < 0.05$

shoots and roots of treated plants were significantly higher than in control plants (+21 ± 6.5 % and +115 ± 12.6 % in shoots and roots, respectively). Indeed, compared to controls, plants treated by seaweed extract showed an increase in nitrate uptake (Fig. 4b) at day 1 and day 30 (+37 ± 11.3 and +38 ± 5.7 %, respectively). Because these last two data points were of the same order of magnitude as the increase in DW (Fig. 1), these results show that N uptake followed the stimulation of growth. In the meantime (Fig. 4c), the *BnNRT2.1* expression level was increased by 7.1 ± 3.5-fold in 1-day treated plants and remained overexpressed at day 30, whereas *BnNRT1.1*

Table 5 Partial list of differentially expressed root genes involved in photosynthesis and nitrogen and sulfur metabolism after 3 or 30 days of treatment with AZAL5 extract

Pathway	GenBank accession no.	Description	Roots 3 days		Roots 30 days	
			Corrected *p* value	Fold change	Corrected *p* value	Fold change
Photosynthesis	AY433944	Br—early light inducible protein (ELIP)	**0.011**	**20.081**	**0.012**	**18.678**
	CP002684	At—ferredoxin-2, chloroplast precursor	**0.016**	**8.113**	**0.013**	**14.416**
	DQ244819	At—ferredoxin-2, chloroplast precursor	**0.021**	**8.321**	**0.017**	**15.319**
	AC189413	Gt—photosystem II reaction center protein Z	**0.011**	**5.518**	**0.013**	**5.380**
	GQ200740	At—phytoene synthase, chloroplast precursor	**0.046**	**6.244**	**0.022**	**6.184**
	EF011647	Th—glucose 6-Pi/Pi transporter	**0.014**	**10.221**	–	–
	NP174327	At—carbonic anhydrase 1	–	–	**0.010**	**5.151**
	L31936	Bc—chloroplast photosystem II 10-kDa polypeptide	–	–	**0.018**	**6.423**
	NM178694	At—cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor	–	–	**0.032**	**6.426**
	AB333800	At—glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor	–	–	**0.037**	**6.397**
	NM102871	At—photosystem I reaction center subunit III (PSAF)	–	–	**0.023**	**5.075**
	AJ245630	At—photosystem I reaction center subunit V	–	–	**0.026**	**7.187**
	U92504	Br—photosystem I reaction center subunit VI	–	–	**0.031**	**5.452**
	NM104102	Br—photosystem I reaction center subunit VI	–	–	**0.036**	**5.014**
	DQ296179	At—photosystem I reaction center subunit psaK	–	–	**0.014**	**6.474**
	DQ296179	At—photosystem I reaction center subunit psaK	–	–	**0.021**	**6.226**
	DQ296179	At—photosystem I reaction center subunit psaK	–	–	**0.025**	**5.403**
	DQ245799	Bc—photosystem II 10 kDa polypeptide, chloroplast precursor	–	–	**0.043**	**5.496**
	L31936	Bc—photosystem II 10 kDa polytpeptide	–	–	**0.018**	**6.368**
	NM111021	Nt—phosphate/phosphoenolpyruvate translocator	–	–	**0.048**	**5.217**
	NM102940	Ta—phosphoribulokinase, chloroplast precursor	–	–	**0.013**	**27.104**
	AF326879	At—plastocyanin major isoform, chloroplast precursor	–	–	**0.036**	**20.861**
	AY07103	At—PSI type III chlorophyll a/b-binding protein	–	–	**0.026**	**7.651**
	BT000613	At—ribulose bisphosphate carboxylase/oxygenase activase	–	–	**0.035**	**7.398**
	NM100952	At—alpha-glucan water dikinase 1, chloroplast precursor	*0.012*	*−6.987*	*0.019*	*−6.566*
	DQ118104	Bn—plastid division regulator MinE	*0.039*	*−18.782*	*0.028*	*−35.693*
	NM124082	At—chlorophyll *a*/*b*-binding protein family (lil3)	–	–	*0.013*	*−5.957*
	NM119135	At—choline monooxygenase, chloroplast precursor	–	–	*0.021*	*−15.831*
	NM113585	Bc—light-regulated protein-like protein	–	–	*0.045*	*−7.913*
	NM101688	At—nudix hydrolase homolog (NUDT4)	–	–	*0.018*	*−5.788*
Nitrogen metabolism	NP181221	Bn—gamma tonoplast intrinsic protein	**0.014**	**8.038**	**0.015**	**6.710**
	NM121822	At—glutamate dehydrogenase 1	**0.017**	**8.031**	–	–
	AB014076	Bn—serine decarboxylase	**0.020**	**8.289**	–	–
	CN729283	At—cysteine proteinase	–	–	**0.027**	**7.700**
	NM105992	Bu—kunitz family trypsin and protease inhibitor	*0.032*	*−12.815*	*0.035*	*−7.164*
	ES266717	Cp—type I proteinase inhibitor-like protein	*0.019*	*−41.481*	*0.017*	*−78.478*
	NP00107809	At—adenosylmethionine decarboxylase	*0.031*	*−5.528*	–	–
	AK226564	At—hydroxypyruvate reductase	*0.014*	*−9.231*	–	–
	BT000269	At—branched-chain alpha keto-acid dehydrogenase E1 alpha subunit	–	–	*0.021*	*−5.193*

Table 5 continued

Pathway	GenBank accession no.	Description	Roots 3 days		Roots 30 days	
			Corrected *p* value	Fold change	Corrected *p* value	Fold change
	NM119466	At—coclaurine *N*-methyltransferase	–	–	*0.016*	*−5.598*
	AF454959	At—cysteine proteinase RD19A	–	–	*0.014*	*−5.980*
	AY559319	Bj—glutamate decarboxylase 4a	–	–	*0.015*	*−5.715*
	AK317177	At—glyoxalase I protein family	–	–	*−0.017*	*−9.353*
Sulfur metabolism	AF411209	Bn—glutathione peroxidase	**0.044**	**8.897**	**0.012**	**9.529**
	AJ311388	Bn—high-affinity sulfate transporter (ST2)	**0.015**	**5.722**	**0.011**	**4.276**
	DQ091257	Bn—low-affinity sulfate transporter	**0.010**	**11.372**	**0.011**	**17.035**
	NM106018	At—phosphoethanolamine *N*-methyltransferase 1	**0.012**	**5.025**	–	–
	NP187918	At—serine acetyltransferase (SERAT2.2)	**0.016**	**9.641**	–	–
	AY299479	Bj—glutathione *S*-transferase 4 (GSTF4)	*0.014*	*−6.942*	*0.022*	*−9.916*
	NM105660	At—glutathione *S* transferase TAU12 (GSTU12)	*0.013*	*−7.094*	*0.010*	*−8.710*
	NM104662	At—glutathione *S* transferase TAU16 (GSTU16)	*0.036*	*−12.154*	*0.021*	*−9.891*
	NM121231	At—glutaredoxin-C10	*0.021*	*−9.091*	–	–

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Positive fold change indicates that the gene is specifically overexpressed in response to seaweed extract (*bold*); negative fold change indicates that the gene is specifically repressed in response to seaweed extract (*italic*)

– indicate genes with expression levels that are not significantly different from control

p values are Bonferroni-corrected. Genes were considered as differentially expressed at $p < 0.05$

expression was more strongly induced later on (67.4 ± 47.4- and 159.6 ± 35.7-fold at 3 and 30 days, respectively, Fig. 4c). Compared to control plants, NR_{act} (Fig. 4d) increased significantly in shoots after 30 days (+51 ± 15.3 %) of treatment but was not affected in roots. However, no nitrate accumulation in shoot or root tissues was detected in plants (data not shown).

S Metabolism

The total S amount in treated plants (Fig. 5a) showed that treatment with AZAL5 resulted in significantly higher shoot (+63 ± 27.1 %) and root (+133 ± 36.3 %) S contents after 30 days of treatment. Unlike N, these data had a greater increase in magnitude than DW (Fig. 1), reflecting a stimulation of sulfate uptake per se (Fig. 5b). Indeed, the total sulfate content in the plants (Fig. 5b) showed that treatment with AZAL5 resulted in accumulation of sulfate in shoots (+48 ± 9.3 % and +28 ± 10.6 % at 3 and 30 days, respectively) and in roots (+19 ± 2.2 % and +24 ± 8.6 % at 3 and 30 days, respectively). In the meantime, compared to control plants, *BnSultr1.1* was upregulated (7.5 ± 3.4-fold) after only 1 day of treatment, whereas *BnSultr1.2* was strongly induced (44 ± 6.7-fold) after 3 days of treatment (Fig. 5c). After 30 days, expression levels of both sulfate transporters were similar between treated and control plants. The expression levels of the *BnSultr4.1* and *BnSultr4.2* genes (Fig. 5d), which encode tonoplastic transporters involved in vacuolar fluxes of sulfate, were induced after only 3 and 30 days of treatment (6 ± 3.3- and 2.7 ± 0.7-fold, 19.5 ± 3.9- and 2.3 ± 0.7-fold at 3 and 30 days, respectively).

Photosynthesis

At days 15 and 21 (Fig. 6a), plants treated with AZAL5 showed an approximately 8 % increase in chlorophyll relative content compared with controls (+7.8 ± 2.1 % at 15 days and +8.9 ± 4.0 % at 21 days). Only one significant difference was found for the net photosynthetic rate after 30 days of treatment (−22 ± 2.2 % for AZAL5-treated plants, Fig. 6b). Microarray analysis, chlorophyll

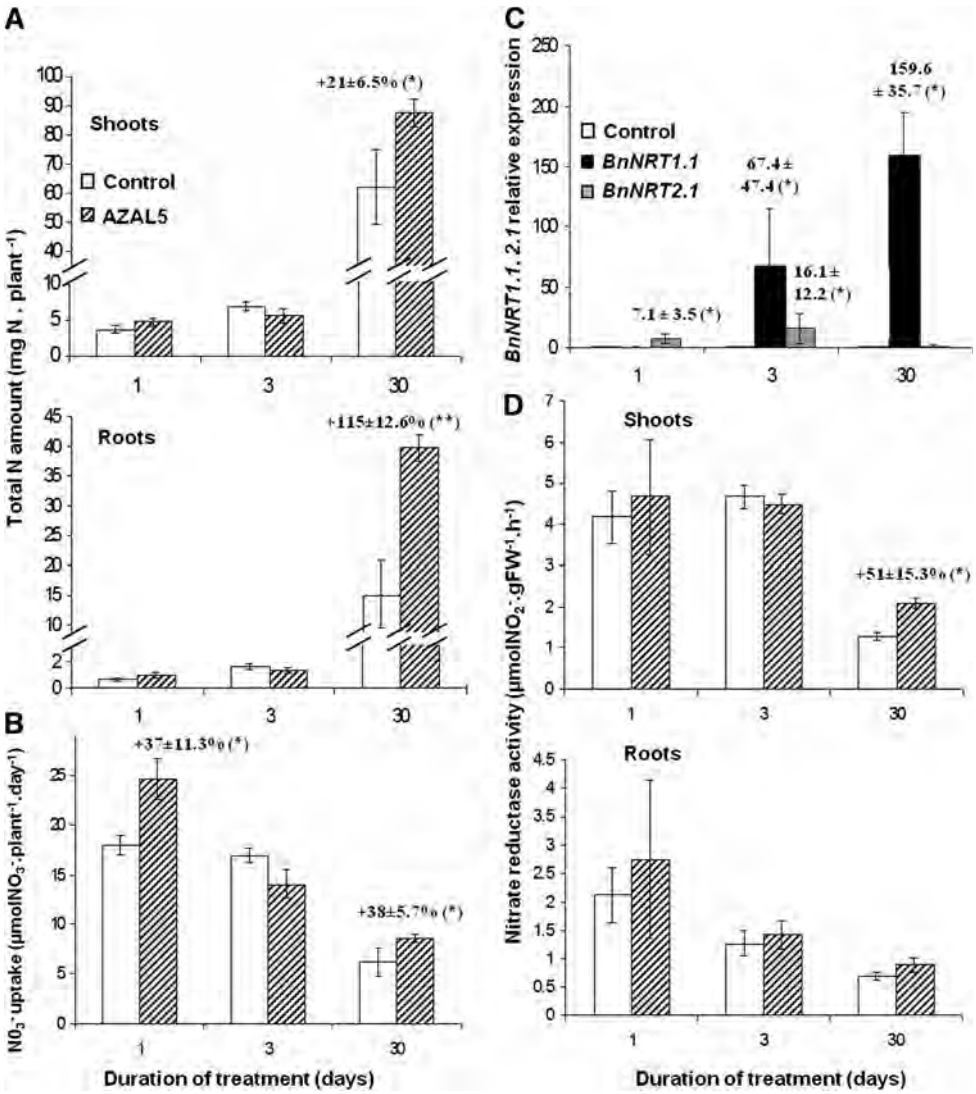

Fig. 4 Effects of AZAL5 extract on N metabolism. **a** Total N amount in shoots and roots of control plants (*white histogram*) or plants treated with AZAL5 (*hatched histogram*). **b** Net nitrate uptake. **c** q-PCR analysis of the expression level of *BnNRT1.1* (*black histogram*) and *BnNRT2.1* (*gray histogram*), two genes encoding root transporters implicated in N uptake. For q-PCR analysis, control (*white histogram*, value 1) corresponds to the expression level of each gene in control plants at each time of harvest. **d** In vivo nitrate reductase activity in shoots and roots of control (*white histogram*) and treated plants (*hatched histogram*). For all data, indicated values are means and *vertical bars* indicate ±standard deviation for $n = 10$ when exceeding the symbol. Significant differences at $^{*}p < 0.05$ and $^{**}p < 0.01$

content, and net photosynthetic rate gave contradictory results. Thus, confocal microscopy was used to observe chloroplast numbers in mature leaf cells from treated or control plants (Fig. 6c). AZAL5 application significantly increased the number of chloroplasts per cell. Indeed, in cells from control plants, the number of chloroplasts was relatively constant during the experiment (39.6 ± 2.1, 47.4 ± 3.7, and 46.6 ± 1.3 at 1, 3, and 30 days, respectively). In leaf cells from treated plants, the number of chloroplasts was always higher than controls whatever the duration of treatment (62.4 ± 3.2, 69.0 ± 4.2, and 113.7 ± 21.3 at 1, 3, and 30 days, respectively). To estimate the potential seaweed effect on chloroplastic ultrastructure, TEM was performed after 30 days of treatment (Fig. 6d). Comparing ultrastructure of chloroplasts from control plants, TEM showed no effect from the application of AZAL5 on thylakoids and no grana organization, but it did show an enhancement of the number and the size of starch granules.

Phytohormone Content Analysis

To verify whether phytohormones could be responsible for this biostimulant action, the phytohormone content of AZAL5-treated rapeseed was determined and compared with that of nontreated rapeseed. Despite the presence of IAA and ABA in AZAL5 extract (Table 2), no significant difference was found in the content for these two phytohormones between treated and nontreated rapeseed (data not shown). Focusing on cytokinins (Fig. 7), AZAL5 treatment resulted in significantly lower content in *trans*-zeatin riboside (tZR, Fig. 7b, approximately −40 % in shoot after 1 and 3 days of treatment and −42 % in root after 3 days) and in *cis*-zeatin riboside (cZR, Fig. 7c, approximately −20 % in root whatever the duration of treatment). Zeatin content (Z, Fig. 7a) shows significant variation only in shoots with a higher content at 3 days and a lower content at 30 days for the AZAL5-treated rapeseed compared with the nontreated rapeseed. Isopentenyladenosine content (iPR, Fig. 7e) in roots followed

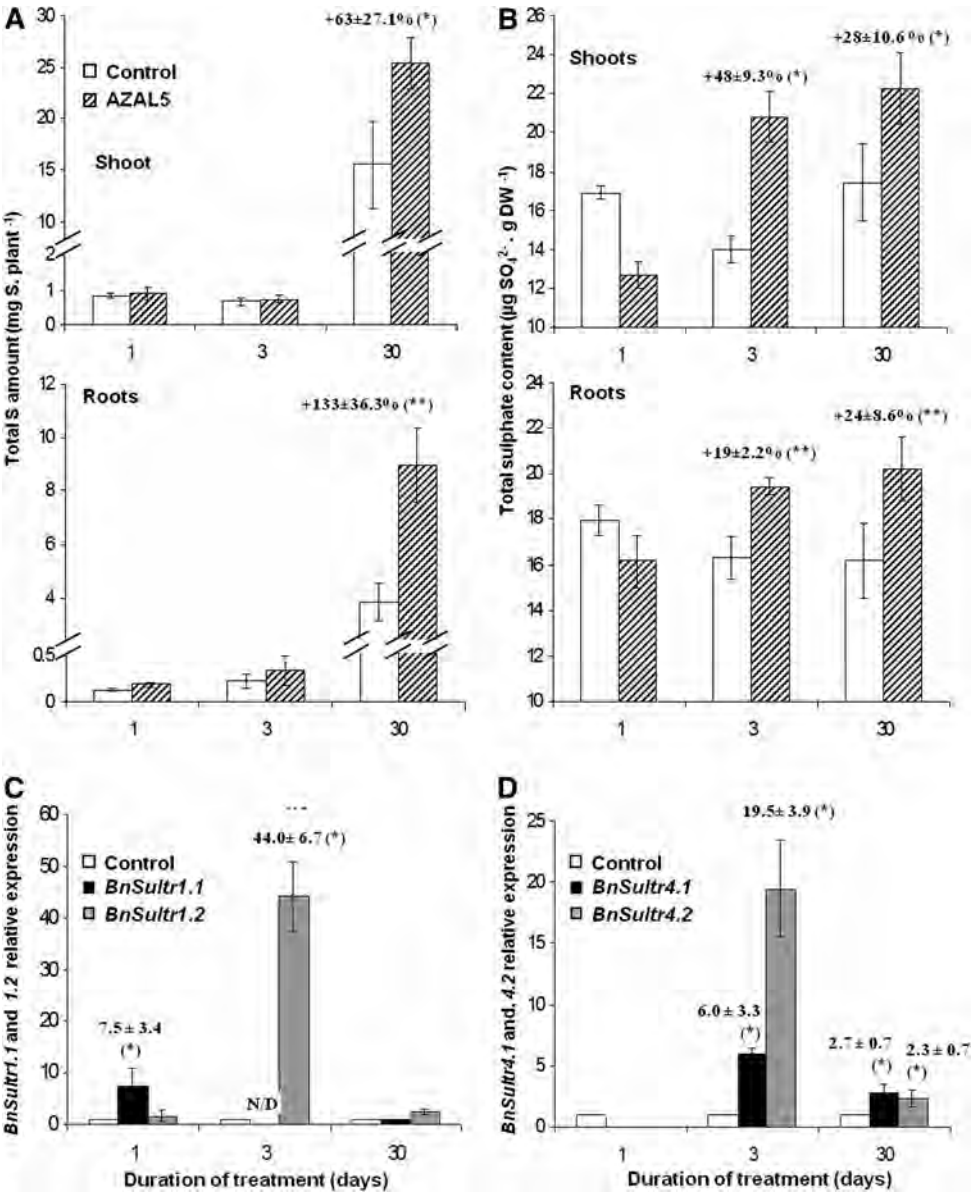

Fig. 5 Effects of AZAL5 on S metabolism. **a** Total S amount in the shoots and roots of control plants (*white histogram*) or treated plants (*hatched histogram*) with AZAL5. **b** Total sulfate content in the shoots and roots of plant controls or treated with AZAL5. **c** q-PCR analysis of the expression level of *BnSultr1.1* (*black histogram*) and *BnSultr1.2* (*gray histogram*), two genes encoding transporters involved in S uptake in roots. **d** q-PCR analysis of the expression level of *BnSultr 4.1* (*black histogram*) and *BnSultr4.2* (*gray histogram*), two genes encoding sulfate transporters involved in sulfate sequestration in shoot vacuoles. For q-PCR analysis, control (*white histogram*, value 1) corresponds to expression level of each gene in control plants at each time of harvest. For all data, indicated values are means and *vertical bars* indicate ±standard deviation for $n = 10$ when exceeding the symbol. Significant differences at $^{*}p < 0.05$ and $^{**}p < 0.01$. N/D, not detected

a similar pattern as Z: a higher content at 1 day and a lower content at 30 days for treated rapeseed compared with the nontreated rapeseed. Isopentenyladenine (iP), one of the main cytokinins in the AZAL5 extract (Table 2), was strongly increased in roots by AZAL5 treatment (Fig. 7d). Indeed, after 1 and 3 days of contact with the extract, iP content in treated rapeseed reached +579 and +594 %, respectively, of the content of nontreated rapeseed in roots. After 30 days of contact with AZAL5, iP content in roots of treated rapeseed still reached +164 % of the content of nontreated plants.

Discussion

To identify AZAL5 metabolic targets in plants, we used microarray analysis specific to *B. napus* that allowed the analysis of 31,561 genes. Nevertheless, most of these genes (60 %, Fig. 3) were not identified due to the lack of complete sequencing of the *B.* genome. So, the picture of plant responses to AZAL5 treatment showed by this microarray analysis could be completed by monitoring the progress of function description of the most differentially expressed genes due to sequencing advances. However, from identified genes that accounted for about 40 % of the gene expression analyzed, the expressions of about 1,000 known genes were significantly affected. This high number of differentially expressed genes reflects a massive effect of AZAL5 at the molecular level that touched almost all areas of plant metabolism. Affected genes could be classified in nine clusters covering the major metabolic functions of plants: carbon and photosynthesis, general cell metabolism, fatty acids, nitrogen/sulfur metabolism,

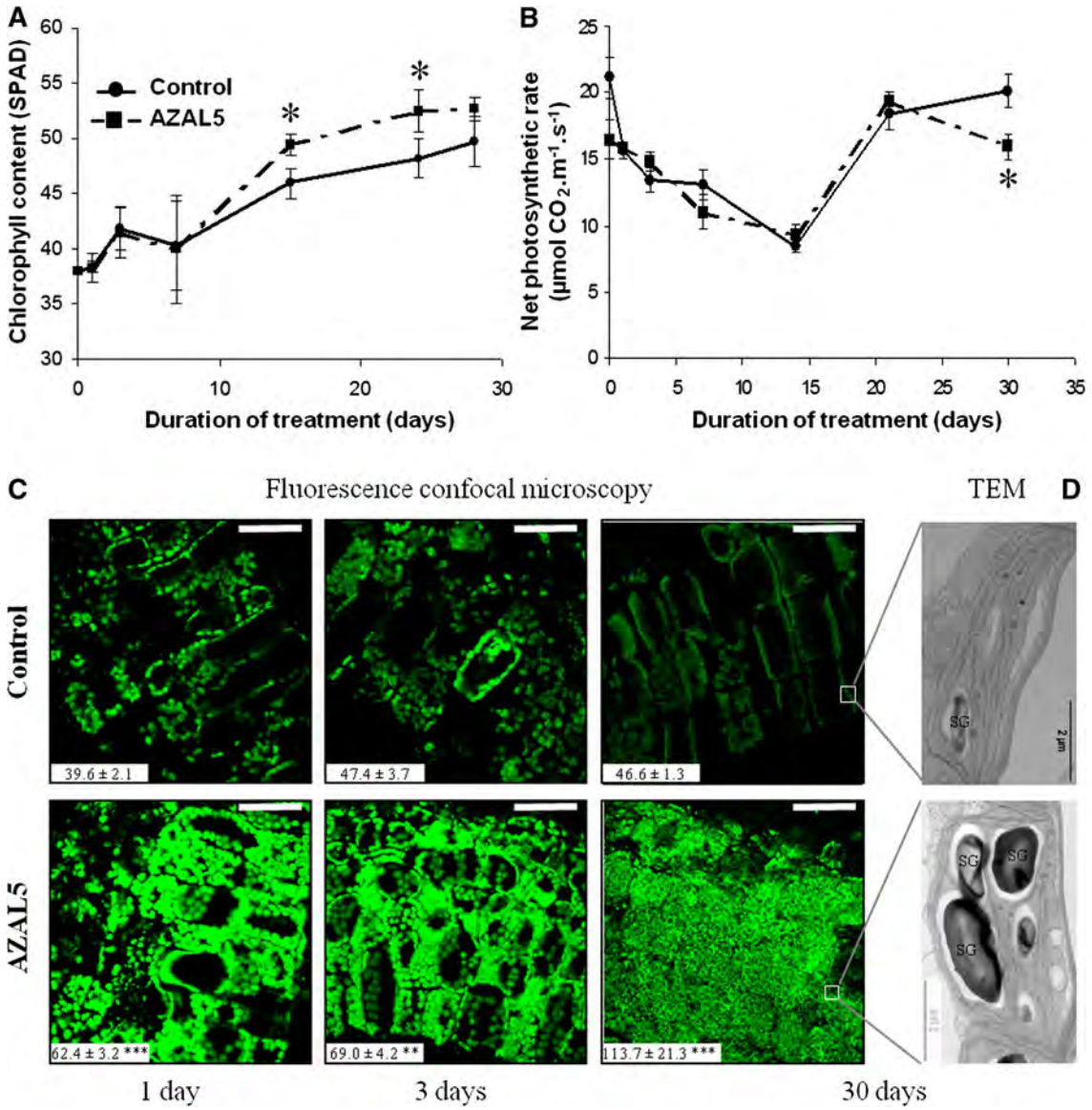

Fig. 6 Effects of AZAL5 on chloroplasts and photosynthesis. **a** Chlorophyll content in leaves from control plants (*solid line*) or treated plants (*dotted line*) measured by SPAD. **b** Kinetics of the net photosynthetic rate from control (*solid line*) or treated leaves (*dotted line*). **c** Fluorescence confocal microscopy of chloroplasts of control or plants treated with AZAL5 after 1, 3, and 30 days of treatment. *Scale bars* = 50 µm. Numbers of chloroplasts per cell are indicated in the *white square* and are the mean ± standard deviation for $n = 10$ cells. **d** Transmission electron microscopy (TEM) observations of chloroplasts from control (*top*) or from leaves treated with seaweed extract (*bottom*) over 30 days. *Scale bars* = 2 µm. *SG* starch granules. For **a** and **b**, results are mean ± standard deviation for $n = 5$ leaves with 10 measurements per leaf. Student's *t* test at $^{*}p < 0.05$,$^{**}p < 0.01$, and $^{***}p < 0.001$

phytohormones, plant development, senescence, responses to stress, and transport of ions and water. Among these pathways, the most affected by AZAL5 application involved carbon and photosynthesis, cell metabolism, and nitrogen and sulfur and responses to stress (Fig. 3). Many studies in the literature have already focused on the nitrogen and sulfur responses to stress, but mostly only from a physiological point of view (Durand and others 2003; Mercier and others 2001; Cluzet and others 2004) and in response to different seaweed extracts. As we used an extract different from those reported in the literature, the effects could also be different. We chose to focus our study on photosynthesis and nitrogen and sulfur metabolism and to corroborate microarray analysis by physiological analyses, but also with more precise q-PCR analyses of selected genes.

Microarray analysis highlighted the enhancement of gene expression related to N and S metabolism and, more particularly, identified genes that encode proteins involved in uptake and assimilation (Tables 4, 5). Physiological

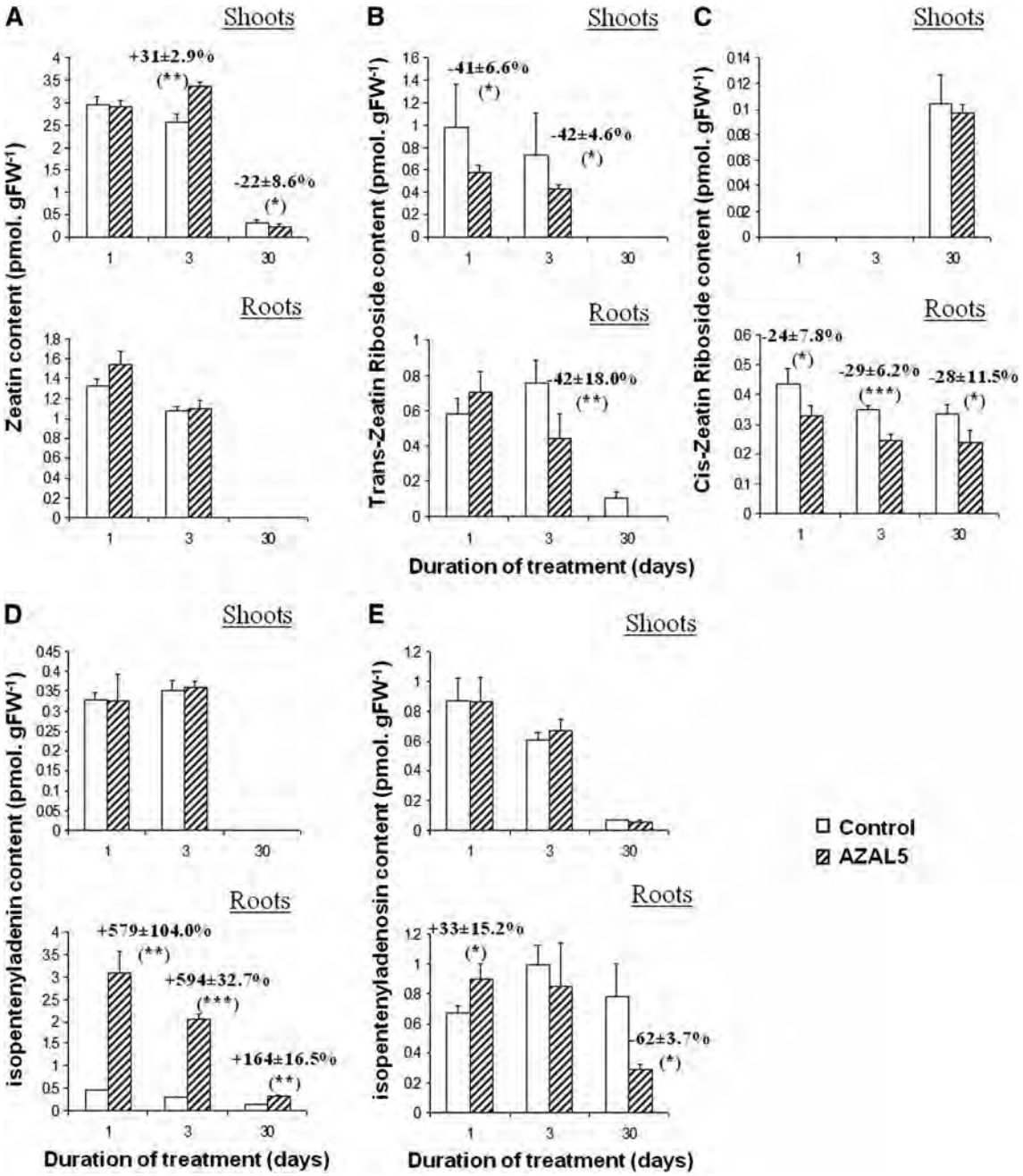

Fig. 7 Cytokinin contents in shoots and roots of AZAL5-treated and control rapeseed plants. **a** Zeatin (Z). **b** *trans*-Zeatin riboside (tZR).**c** *cis*-Zeatin riboside (cZR). **d** Isopentenyladenine (iP).**e** Isopentenyladenosine (iPR). For all data, *vertical bars* indicate standard deviation for $n = 3$. Significant differences at $^{*}p < 0.05$ and $^{**}p < 0.01$. Cytokinins benzyladenine (BA), benzyladenine riboside (BAR), meta-topolin (mT), meta-topolin riboside (mTR), ortho-topolin (oT), ortho-topolin riboside (oTR), dihydrozeatin (DHZ), and dihydrozeatin riboside (DHZR) were not detected in the samples (data not shown)

analyses also gave access to more precise interpretation of data. For N metabolism (Fig. 4), increases in NO_3^- uptake (+38 ± 5.7 %), N content (+21 ± 6.5 and 115 ± 12.6 % in shoots and roots, respectively), and enhancement of NR enzymatic activity were in the same range of magnitude as the DW increase (+23 ± 8.6 and 102 ± 13.4 % in shoots and roots, respectively). Moreover, q-PCR analysis of expression of *BnNRT1.1* and *BnNRT2.1* genes which encode nitrate transporters showed an induction of these genes in roots of treated plants. Surprisingly, these changes were not revealed by microarray analysis. However, nitrate transporters are a very large family of genes (Daniel-Vedele and others 1998), and a BLAST analysis showed that the nitrate transporter probe used on microarray

analysis is weakly specific for the *BnNRT1.1* and *BnNRT2.1* isoforms. All these data suggest that supplemental N taken up by roots of treated plants was directly assimilated in relation to the growth rate without being stored (no N or nitrate content enhancement, data not shown). It must be pointed out that expression of *NRT1.1* was strongly induced by AZAL5, whereas recent results suggest (Castaings and others 2011; Krouk and others 2010) that this gene may have a role in N sensing as well as in auxin transport, and thus have an effect on lateral root growth, a process that seems also to be largely stimulated by AZAL5, as suggested by the large increase of root DW (Fig. 1c). An increased expression of the main roots and tonoplastic sulfate transporter genes (Fig. 5) and an enhancement of assimilatory gene expression were also found (Table 4). However, these results are not in full agreement with previous works that showed an accumulation of N or nitrate in response to seaweed extract treatment (Nelson and Van Staden 1984; Turan and Köse 2004; Mancuso and others 2006) of grapevine, cucumber, and *A. thaliana*. The enhancement of NR_{act} found in this study has already been reported in *A. thaliana* (Durand and others 2003). As N is not increased by addition of AZAL5 to the nutrient solution (Table 1), enhancement of N uptake in response to treatment is possibly a consequence of growth promotion. For S metabolism (Fig. 5), S content after 30 days (+63 ± 27.1 and 133 ± 36.3 % in shoots and roots, respectively) increased more than dry matter accumulation and this resulted in sulfate accumulation (+28 ± 10.6 and 24 ± 8.6 % in shoots and roots, respectively). Rathore and others (2009) demonstrated that soybean accumulates S and N in its seeds in response to seaweed extract treatment. These results are in accordance with our study where AZAL5 seems to have a direct and early effect on sulfate uptake, assimilation, and storage. The latter suggests that S metabolism was increased more than the growth rate and that the S supply exceeded demand for growth.

The second axis of our study focused on carbon assimilation by photosynthesis. In our work, significant effects on chlorophyll content and net photosynthesis were found late in the response to AZAL5 treatment (Fig. 6), whereas enhancement of chlorophyll content has been reported in grapevine and strawberry in response to seaweed extract (Mancuso and others 2006; Sivasankari and others 2006; Spinelli and others 2010). However, microscopic observations (Fig. 6) show that in response to AZAL5, chloroplasts produce higher quantities of starch compared with non-treated control plants. This result suggests that AZAL5 has no effect on the clear phase of photosynthesis (net photosynthesis measurement at PAR 1000), but it seems to enhance the dark phase of photosynthesis (carbon fixation and starch synthesis). To support this hypothesis, microarray results showed that genes involved in carbon fixation, such as Rubisco or carbonic anhydrase, were mostly upregulated by AZAL5 treatment (+5.666-fold for carbonic anhydrase in shoots after 3 days; + 5.507-fold and +5.151-fold for carbonic anhydrase after 30 days in shoots and roots, respectively; +7.398-fold for Rubisco in roots after 30 days) (Tables 4, 5). Rubisco is known for its involvement in the Calvin cycle for carbon fixation (Cardon and Berry 1992). Moreover, carbonic anhydrase, an enzyme that requires zinc, catalyzes the reversible hydration of carbon dioxide, thus facilitating its transfer and fixation (Ramanan and others 2009). Carbonic anhydrase is important in photosynthesis and respiration as it participates in the transport of inorganic carbon to actively photosynthesizing cells and away from actively respiring cells (Moroney and others 2001). This suggests that carbonic anhydrase and Rubisco have complementary activities, with carbonic anhydrase hydrating and transferring CO_2 to Rubisco, which fixes it into glucose and then starch. Therefore, enhanced gene expressions of these two proteins showed by microarray analysis could explain the increased carbon fixation that was suggested by microscopy observation due to the large number of starch granules in AZAL5-treated plants (Fig. 6d).

Furthermore, confocal microscopy observations showed an important enhancement of the number of chloroplasts per cell after 1 day of contact with AZAL5 extract (Fig. 6). Okazaki and others (2009) obtained similar results with *A. thaliana* in response to exogenous application of cytokinins. According to these authors, plastid division components of chloroplasts were under the control of cytokinin-responsive transcription factor. Consequently, application of cytokinins enhances chloroplast division and increases the number of chloroplasts per cell. In our study, only one plastid division component was found on the microarray, the plastid division regulator *MinE*, but it was clearly downregulated in response to AZAL5 treatment after both 3 and 30 days of contact. However, the literature on this gene gives contradictory results. Itoh and others (2001) showed that overexpression of *MinE* in *A. thaliana* resulted in cells containing only one giant chloroplast. Kojo and others (2009) obtained the same phenotype with an *AtMinE*-deficient mutant (2,000-fold reduced *MinE* expression compared with wild type). The second aspect of the Okazaki study (2009) was the role of exogenous cytokinin in chloroplast division. Cytokinins have long been reported for their protective effects on chloroplasts (Zavaleta-Mancera and others 2007) and for enhancing the chloroplast division (Okazaki and others 2009). In our study, we show a six fold enhancement of iP content in root (Fig. 7). As iP and ABA contents in AZAL5 extract were similar (Table 2), but only cytokinin content in plants was enhanced by the treatment, this suggests an uptake of iP

from the AZAL5 extract and/or a de novo synthesis of iP as an early response (1 day) to AZAL5 treatment. However, it must be pointed out that Matsumoto-Kitano and others (2008) showed that iP-type cytokinins were synthesized mainly in the shoot and translocated from the shoot to the root via the phloem. This early synthesis of cytokinin could explain the early activation of chloroplast division, as suggested by Okazaki and others (2009), and could also explain the enhancement of nutrient uptake as Collier and others (2003) reported that iP enrichment in roots of *Fagus sylvatica* caused an increased expression of a high-affinity nitrate transporter. However, iP has no effect on sulfur metabolism, although zeatin increased transcript accumulation of sulfur-responsive genes (Ohkama and others 2002).

These results suggest that the first event to occur after AZAL5 application on plants was the increase in chloroplast number per cell (Fig. 6), in parallel with the increase in iP concentration (Fig. 7) and the enhancement of the expression of genes encoding root nitrate and sulfur transporters [especially *NRT2.1* ($\times 7.1 \pm 3.5$ after 1 day, Fig. 4) and *BnSultr1.1* ($\times 7.5 \pm 3.4$ after 1 day, Fig. 5)]. These first responses were followed by sulfate accumulation in shoots and an even greater number of chloroplasts over the duration of the treatment, suggesting that these early events were the preliminary effects that contribute to the global change in plant metabolism observed from 3 to 30 days of treatment with AZAL5. Surprisingly, this increase in chloroplast number is not associated with an increase in net photosynthetic activity (Fig. 6a). However, because chloroplasts were the first organelle affected during the senescence process (Smart 1994), it could be assumed that the increase in the number of chloroplasts in AZAL5-treated plants would promote a delay in senescence and extend the life span of leaves. Previous work has shown that for WOSR, an increase in the life span of leaves would allow a better synchronization between N remobilization associated with leaf senescence and seed filling (Rossato and others 2001; Malagoli and others 2005) and improved NUE of the WOSR crop. This hypothesis is also supported by microarray analysis that reveals upregulation of some genes encoding protease inhibitors and downregulation of stay green protein (Srg), which are able to limit leaf protein degradation (Etienne and others 2007; Desclos and others 2008) and protect chlorophyll degradation (Park and others 2007) during leaf senescence, respectively. According to this work, it could be assumed that chloroplast modifications could act on the duration of leaf senescence and the leaf life span and allow improvement in the NUE of WOSR. Thus, the seaweed extract reported here could be especially relevant to augment or substitute for fertilizers actually used and improve the agroenvironmental balance of WOSR.

This study, which combined microarray and physiological analyses to explain effects on WOSR growth, gives clues about the metabolic targets of *A. nodosum* (Linnaeus) Le Jolis seaweed extract, AZAL5. Physiological analyses confirmed microarray results of the activation of nitrogen and sulfur absorption and assimilation. Enhancement of N, C, and S assimilation could explain increased growth of plants treated with AZAL5. Furthermore, microscopy observations showed a clear and early effect of seaweed extract on the chloroplast number per cells and starch granules. However, enhanced nutrient assimilation alone could not account for the total improvement in WOSR agroenvironmental balance, so exploration of nutrient remobilization should be undertaken. Furthermore, some genes involved in different metabolic pathways (such as responses to stress and senescence) were up- or downregulated in response to AZAL5 treatment (supplemental data, Supplementary Tables 1 and 2) and remain to be studied to improve our knowledge of the effects of seaweed extract on plant physiological processes.

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Seaweed extracts as possible agents in improving the emergence of barley, oats and maize in Northern Ireland

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Introduction A combination of factors such as rising fertiliser prices, precarious world food supplies, climate change and restrictions on pesticide usage (due to resistance problems and EU regulation) has led to pressures on agricultural scientists to examine more sustainable options. One potential area is that of biostimulants, products which stimulate the plant's own defence systems to allow it to cope better with stress induced by such factors as salinity, drought, pests, diseases and temperature extremes. One of these, temperature, has particular relevance to N. Ireland where low temperatures in the spring affect the growth and emergence of forage maize. The area of the crop has expanded in recent years, but further expansion is constrained by the high cost of either growing under polythene or waiting until the frost risk has passed. Faster crop emergence could also help to suppress weed growth of temperate cereals, such as oats and barley, when grown under organic conditions. This paper reports on initial attempts to use extracts (biostimulants), derived from seaweed, to improve emergence in barley, oats and maize.

Material and methods In the first experiment, seeds of barley (cv. Westminster) and oats (cv. Firth) were either soaked for 18 h in water or a range of seaweed extracts (obtained from *Palmaria palmata*, *Delesseria sanguinea*, *Porphyra* sp., *Laminaria* sp. or *Ulva lactuca* by maceration in cold water) or in distilled water or left unsoaked. Ten seeds were then sown out in each of 15 cm diam. pots filled with peat-based compost, placed in a growth cabinet with 12 h light; 12h dark at 15°C and observed for emergence (out of 10). There were five replicates. In the second experiment, maize seeds (cv. Goldcob) were similarly treated, but with a range of commercially available seaweed products (Algifol (Neomed Pharma GmbH), Algaegreen (Oilean Glas Teo, Co. Donegal), Ecolicitor and Nematec (Bioatlantis Ltd., Co. Kerry)). Pots were placed in a growth cabinet at 10°C and again observed for emergence. There were six replicates. All data were analysed by Analysis of Variance using Genstat version 12.1.

Results In the first experiment, barley seedlings, grown at 15°C, emerged significantly more quickly following treatment with a range of seaweed products than when treated with either distilled water (water control) or left untreated (dry control) (Fig. 1). Although results for oats were in a similar direction they were not significant. Maize, grown at 10°C, germinated significantly faster when it had been pre-treated with a range of commercial seaweed extracts compared with water and dry controls (Fig,. 2).

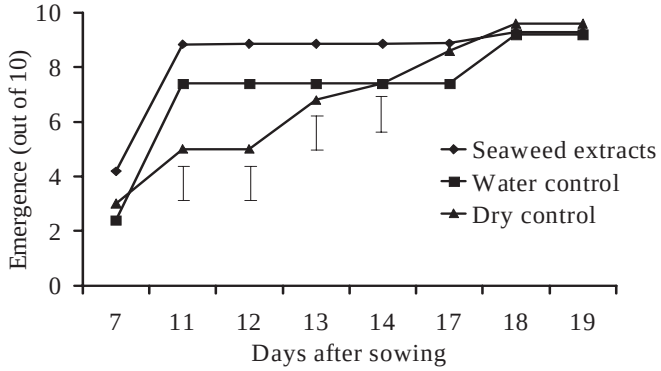

Figure 1 Effect of seaweed extracts (meaned over products) on emergence of barley seedlings at 15°C. L.s.d. at 5% for comparison between extracts and controls

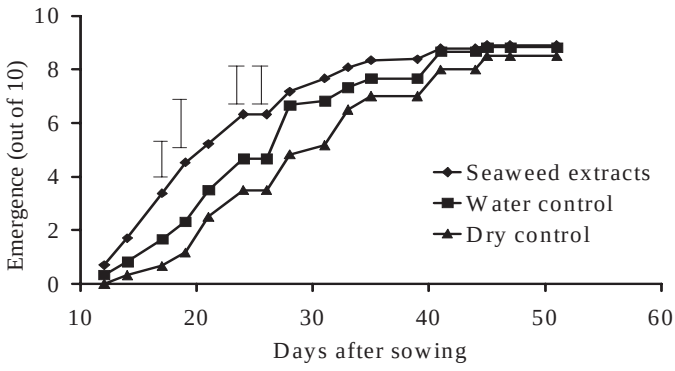

Figure 2 Effect of seaweed extracts (meaned over products) on emergence of maize seedlings at 10°C. L.s.d. at 5% for comparison between extracts and controls

Conclusions These results indicate some potential for enhancement of emergence of a range of cereals with extracts obtained from seaweeds, and are similar to those obtained by Farooq *et al*. (2008), who found that priming maize seeds with salicylic acid improved emergence and uniformity both at its optimal temperature for growth (27°C) and at 15°C. However, as Khan *et al*. (2009) have indicated, the biostimulatory potential of many seaweed products has not been exploited due to lack of scientific data on growth factors and their mode of action. Further basic research is therefore needed before this potentially valuable bioresource could be exploited commercially as an emergence promoter.

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1. Assessment of Super *Fifty* and Ecolicitor to Improve Cereal Tolerance to Drought Stress.

Barley seeds were established at 5 plants per 1 litre pot (8 pots per treatment) on 27 July 2008 in a 6:3:4 v/v mixture of sieved pasteurised soil, sharp sand, milled peat. Plants were grown in a glasshouse and watered daily until September 10th when half the pots per treatment were subjected to drought stress by omitting watering on alternate days, until harvest of barley on 29 September. Drought stress on wheat stopped then too, but those plants were grown on with normal watering until October 28th. Foliar sprays of products at the rates listed in Table 6.1 were applied on these dates.

Table 6.1: Treatment Schedule for Cereal Drought Stress

Date	Days after sowing	Date	Days after sowing	Comments
05-Aug	9	03-Sep	38	
12-Aug	16	10-Sep	45	

Table 6.2: Effect of BioAtlantis seaweed extract foliar sprays on weight of **BARLEY** plants grown with, or without, a 3 week period of drought stress.

Foliar spray	Fresh weight of Barley shoot per plant (g)				
	Application Rate (L/Ha)	Drought Stress		% Increase Over Control	
		Without	With	Without	With
Super *Fifty*	0.5	20.25*	18.21	11.38	0.55
Super *Fifty*	2.5	19.89	17.33	9.40	-4.31
Ecolicitor	0.5	20.36*	18.32	11.99	1.16
Ecolicitor	2.5	21.52*	19.73	18.37	8.94
Water		18.18	18.11	0	0
LSD (P≤ 0.05)		2.84	2.84		

Results:
No seaweed extract treatment significantly improved growth under drought stress. While the mean fresh weights of all plants treated with one concentration of Ecolicitor was significantly greater than the control.

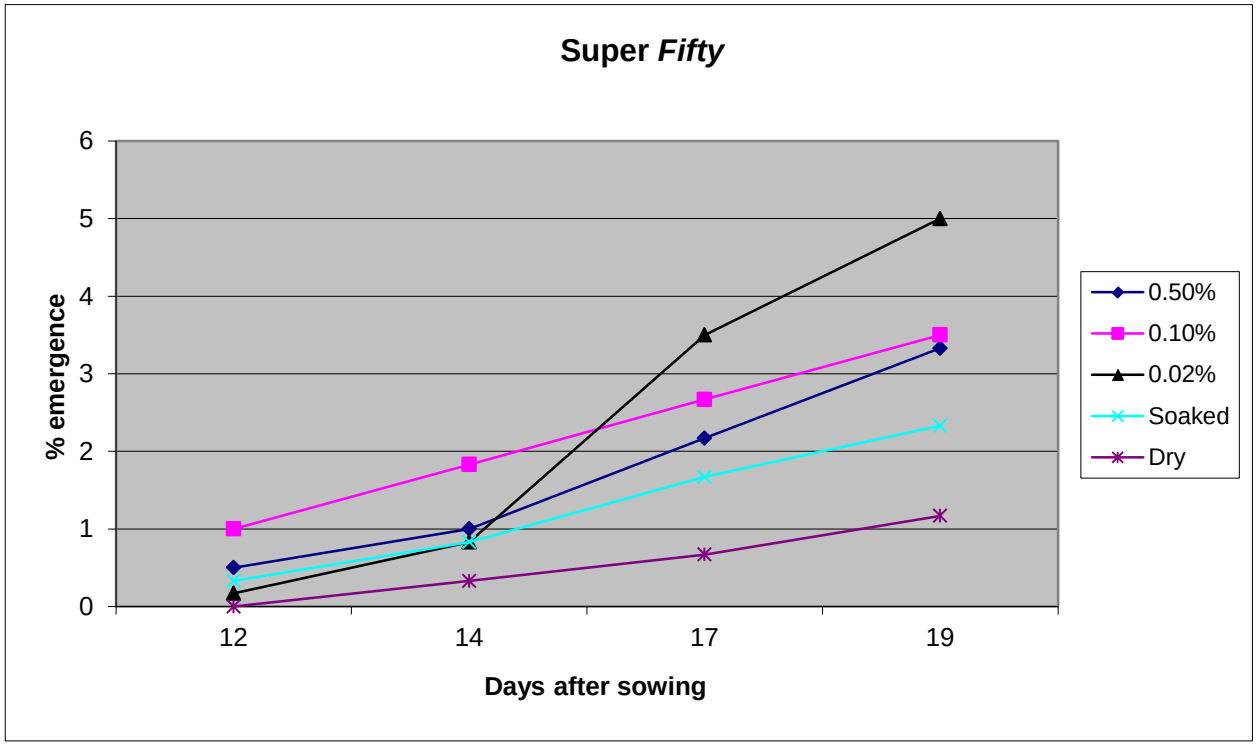
1. Effect of BioAtlantis Ecolicitor and Super Fifty on Maize Seed Germination

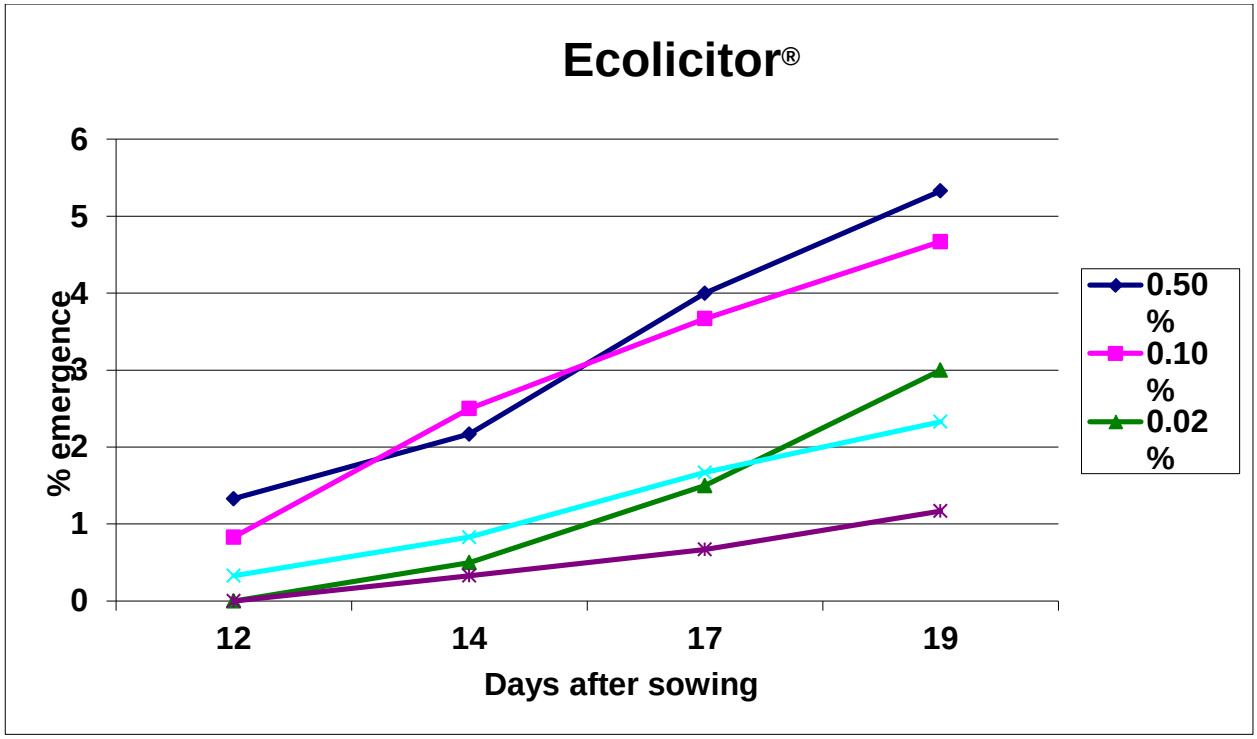
Location: Agri Food and Biosciences Institute (AFBI), Plant Science Department, Belfast, Co. Antrim, Northern Ireland.

Date: These trials were performed and completed between January 2007 and September 2009.

11.1 Trial Summary:

Maize seeds were soaked overnight (18 hrs) in diluted seaweed extract, or water. These seeds were then planted 1-2 cm in depth in soil-based compost. This was watered and placed in growth chamber at 10°C. BioAtlantis Ecolicitor and Super *Fifty* were compared, at 3 dilutions (1 in 200, 1000 and 5000) to a negative control. Controls were water-soaked seeds and seeds planted without prior soaking (Dry). Data presented are for emergence counts done on days 12, 14, 17. and 19. There are 6 reps, each with 10 seeds. **Figure 11.1:** Effect of various concentrations of BioAtlantis Super *Fifty* on Seed Germination in Maize





Results:

Results indicated that from 17 days after sowing, emergence levels were significantly improved in seeds that have been treated with BioAtlantis Ecolicitor and Super *Fifty*. The data indicated that lowest concentration of BioAtlantis Super *Fifty* was optimal. In this case, the highest level of emergence was achieved at a concentration equal to 0.02% where 5% emergence was observed at 19 days post sowing. BioAtlantis Ecolicitor also increased the rate of emergence at an optimal concentration of 0.1%. BioAtlantis Ecolicitor provided a higher rate of early seed emergence by day 14 compared to Super Fifty.

National Organic Standards Board
Crops Subcommittee
Petitioned Material Proposal
Laminarin

+February 26, 2014

Introduction

The NOSB received a petition for Laminarin, a seaweed extract for disease control that is allowed by EPA for that purpose.

Background

From the Laminarin petition:

5. The source of the substance and a detailed description of its manufacturing or processing procedures from the basic component(s) to the final product.

Stage 1: Fresh *Laminaria digitata* seaweed, harvested on the North Brittany coast of France, undergoes extraction in tap water that has a pH adjusted to 2 by addition of sulfuric acid. At this stage sulphuric acid is a processing aid. Laminarin can be extracted at neutral pH or in acidic conditions. The described acidic conditions do not modify the chemical structure of laminarin. The addition of sulfuric acid avoids the co-extraction of other compounds such as alginates (which occurs at neutral pH). When alginates are extracted, the solution has a higher viscosity; purification and filtration steps for laminarin then become much more difficult. This is the reason why sulphuric acid is used to lower the pH and to facilitate the manufacturing process.
Stage 2: The extract is then filtered using a Seitz filter.
Stage 3: The solution then undergoes tangential filtration (membrane technology – physical process) to remove impurities from the solution. The filtrate containing laminarin is kept for the next purification step and the retentate is removed.
Stage 4: The filtrate (see above) then undergoes a second tangential filtration to remove any remaining impurities (filtrate), thereby resulting in a purified solution of laminarin in water (retentate).
Stage 5: The pH is adjusted between 6 and 7 by adding sodium hydroxide to neutralize the acidic solution, resulting in a solution of laminarin at neutral pH for formulation purposes (i.e., Vacciplant formulation). The addition of dilute sodium hydroxide does not modify the chemical structure of laminarin.

From the note from NOP to Crops Subcommittee, 6/3/13:

In NOP's review of the eligibility of this petitioned substance for the National List, we reviewed the manufacturing process against the draft guidance on classification of materials (NOP 5033). Based on our preliminary review, this substance may be classified as nonsynthetic. We have moved this petition forward for NOSB review and final determination on the classification status for the following reasons:

- The classification guidance is currently in draft form
- Other aquatic plant extracts are classified as synthetic for crop production at 205.601(j)(1)
- At this time, NOP is not aware of any products containing laminarin as an active ingredient that are approved by certifying agents or third-party material review organizations, such as EPA or OMRI

Discussion

The Crops subcommittee also reviewed the manufacturing process against the draft guidance on classification of materials (NOP 5033, section 4.6):

> **4.6 Extraction of Nonorganic Materials**
> Some materials are produced using manufacturing processes that involve separation techniques, such as the steam distillation of oil from plant leaves. Separation and extraction methods may include, but are not limited to, distillation, solvent extraction, acid-base extraction, and physical or mechanical methods (e.g., filtration, crushing, centrifugation, or gravity separation).
>
> For purposes of classification of a material as synthetic or nonsynthetic, a material may be classified as nonsynthetic (natural) if the extraction or separation technique results in a material that meets the following criteria:
>
> • At the end of the extraction process, the material has not been transformed into a different substance via chemical change;
> • The material has not been altered into a form that does not occur in nature; and
> • Any synthetic materials used to separate, isolate, or extract the substance have been removed from the final substance (e.g., via evaporation, distillation, precipitation, or other means) such that they have no technical or functional effect in the final product.

The majority of the subcommittee has determined that Laminarin is extracted by an acid-base extraction and meets the criteria in section 4.6 above.

In regards to the third bullet point above the subcommittee majority believes that the acid-base reaction itself neutralizes any of the sulfuric acid starting material to the degree that it has no technical or functional effect. The minority opinion tries to draw parallels between laminarin which is extracted and then used to boost the plant's immune defenses against disease with sulfuric acid used to stabilize manure (a petition that was rejected) or acids used to stabilize fish products listed on §205.601. The majority feels these comparisons are not relevant because of the acid being used in substantially greater quantities in manure and fish, and the fact that they are both fertilizers means that the residual sulfates or phosphates would have a functional effect in the fertilizers. Laminarin is used for disease control at a rate of 0.52 - 1.04 fl. oz. per acre (as stated in the petition) which would not provide a functional effect from some parts per million of that rate being sulfate.

Because of a determination that it is non-synthetic, the subcommittee has not filled out a checklist as it does not need to be added to the National List.

Minority Opinion
A minority of the Subcommittee supported the view that laminarin is synthetic because sulfuric acid is added but not removed. Sodium hydroxide is added to neutralize sulfuric acid, but the sodium sulfate produced by the neutralization reaction (which does not chemically change laminarin) is not removed.

The minority agrees that laminarin does not undergo chemical change in the extraction process. Unfortunately, however, the NOP decision tree is incomplete –it does not cover all of the criteria in the guidance document NOP 5033. The guidance document states, “Some materials may be considered synthetic due to chemical changes which occur during manufacturing, while others substances may be classified as synthetic due to addition of small amounts of synthetic ingredients.”

In the case of laminarin, we need to look at the synthetic ingredients that are added. The section on extraction in the NOP guidance states that in order for a material to by classified as nonsynthetic, "Any synthetic materials used to separate, isolate, or extract the substance have been removed from the final substance (e.g., via evaporation, distillation, precipitation, or other means) such that they have no technical or functional effect in the final product."

The extraction of laminarin involves the addition of sulfuric acid, as described in the petition. The sulfuric acid is not removed. Sodium hydroxide is added to neutralize the acid, but the sulfate remains. While there is no definition of "technical or functional effect" in the NOP regulations or the guidance, our calculations suggest that the amount of sulfate and sodium added to laminarin in the extraction process is significant.

The minority does not claim that the sulfate in laminarin constitutes a synthetic plant nutrient because it is not used in quantities that would be significant nutritionally to plants. Rather, the claim is that the sulfate is a significant residue within the laminarin that is not removed.

Some relevant points that we considered:

1. Sulfuric acid is added during the extraction of laminarin to reduce the pH to 2. Later, it is neutralized with the addition of the base sodium hydroxide. Although the sulfuric acid is neutralized, it is not removed. We calculate that altogether, 624 parts per million (ppm) sulfate and 299 ppm of sodium are added.[1] Because the kelp provides some (unknown) buffering capacity, the quantities are probably somewhat higher than this calculation indicates.
2. The NOSB has previously found (in 2006 and 2012, for anaerobic digestion of livestock and poultry manure) that the addition of sulfuric acid, even when followed by a step that neutralizes the acid, leaves behind a significant synthetic residue that has a functional effect in the agricultural system.
3. OMRI regards sulfuric acid and sulfate as prohibited, with limited specific exceptions.
4. The listing of liquid fish products is an instructive precedent indicating that when pH is adjusted with a synthetic, the product should be classified as synthetic.

Subcommittee Action & Vote

Classification Motion:
Motion to classify Laminarin as nonsynthetic
Motion by: Zea Sonnabend
Seconded by: Harold Austin
Yes: 5 No: 2 Absent: 0 Abstain: 0 Recuse: 0

Because laminarin was classified as non-synthetic, no further action by the Crops Subcommittee is necessary.

Approved by Zea Sonnabend, Subcommittee Chair, to transmit to NOSB February 26, 2014

[1] Compare this concentration to the Secondary Maximum Contaminant Level of sulfate in drinking water and the EU standard for drinking water --both 250 ppm.

Agency Response Letter GRAS Notice No. GRN 000123

CFSAN/Office of Food Additive Safety
July 25, 2003

Michael Russell
Chief Operating Officer
Fuji Foods, Inc.
6206 Corporate Park Drive
Browns Summit, NC 27214

Re: GRAS Notice No. GRN 000123

Dear Mr. Russell:

The Food and Drug Administration (FDA) is responding to the notice, dated January 14, 2003, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on January 22, 2003, filed it on January 28, 2003, and designated it as GRAS Notice No. GRN 000123.

The subject of the notice is *Laminaria japonica* extract, prepared by the extraction of dehydrated *L. japonica* leaves with water. The notice informs FDA of the view of Fuji Foods Inc. (Fuji) that *L. japonica* extract is GRAS, through scientific procedures, for use as a flavor enhancer or flavoring agent in marinades, soups, gravies, and seasonings. The maximum amount of *L. japonica* extract in the final food product would be 0.08 percent, regardless of whether *L. japonica extract* is added directly to food or added to marinades and gravies that are subsequently applied to food; above this concentration, some foods acquire an undesirable taste.

Because *L. japonica* is a type of seaweed, Fuji describes generally available information about consumption of seaweed and seaweed-derived products. Seaweed is a common food in Japan, where it is consumed as a vegetable, made into tea, or present as an ingredient in foods such as soups, noodles, salads, cakes, jellies, and sauces. The mean intake of seaweed in the Japanese diet has been reported to be approximately 7 grams per person per day (g/p/d). Seaweed species that are commonly consumed in Japan include red algae such as nori (*Porphyra* spp.) and brown algae such as kombu (*Laminaria* spp.) and wakame (*Undaria* spp.). Fuji also describes generally available information about "kelp," which is a general term that refers to brown algae, including *Laminaria* spp. and *Macrocystis* spp. Fuji describes seaweed-derived products such as alginates, which are polysaccharides that are isolated from brown algae and added as thickening agents to foods.

L. japonica is a brown algae that is the primary species of seaweed consumed in Japan and is ranked number one worldwide by production volume of aquaculture products. *L. japonica* is prevalent in the cold waters of the North Atlantic and Pacific oceans. With the exception of

the root, the entire plant is used as a whole food or as a source of food ingredients. FDA has affirmed that brown algae (including dried and ground or chopped *L. japonica*) is GRAS for use in spices, seasonings, and flavorings as a flavor enhancer or flavor adjuvant (21 CFR 184.1120).

Fuji describes the general composition of kelp reported in the literature. Kelp dry matter contains approximately 8 percent protein, 8 percent crude fiber, 55 percent carbohydrate, and 27 percent minerals. The carbohydrates in kelp include polysaccharides such as alginates, lamanarins, and fucoidans. Fuji also cites published values for the chemical composition of *L. japonica*, including the volatile chemical components of *L. japonica*. Fuji notes that *L. japonica* essential oil, which is a mixture of aroma compounds obtained by distillation, contains predominantly cubenol (sesquiterpene alcohol), myristic acid, and palmitic acid and that the sesquiterpene alcohol is an important contributor to kelp flavor.

Fuji describes the manufacture of two product forms of *L. japonica* extract - i.e., a liquid form and a powder form. To prepare the liquid form, dehydrated *L. japonica* is soaked in water for a minimum of 16 hours, and then briefly heated to 190 degrees F. The liquid extract is drained, filtered and centrifuged, and then heated to 185 degrees F for at least 5 minutes. Salt is added if needed to adjust the salt content to specification (13 - 14 percent) and the *L. japonica* leaves are discarded. The resulting liquid extract may be used as such or made into a powder by spray drying the extract with a carrier such as maltodextrin.

Fuji reports the chemical composition of liquid *L. japonica* extract, which contains 39 percent dissolved solids and 61 percent water. The solid material includes salt, free amino acids, carbohydrates, and minerals. Fuji presents the amino acid composition of *L. japonica* extract in detail and notes that the predominant amino acids are glutamic acid and aspartic acid. Fuji notes that the component of *L. japonica* extract that is primarily responsible for the flavor enhancing property of the extract is the sodium salt of glutamic acid (i.e. monosodium glutamate). Fuji reports the mineral composition of the extract, which contains sodium, potassium, calcium, magnesium, iron, zinc, and iodine. Fuji also provides specifications for *L. japonica* extract, including a specification for the iodine content (less than or equal to approximately 1600 milligrams iodine per kilogram of extract). Fuji estimates that intake of *L. japonica* extract from its proposed uses would be approximately 150 milligrams per person per day (mg/p/d) at the 90th percentile[(1)] and states that the use of *L. japonica* extract as a flavor enhancer or flavoring agent is self-limiting.

Fuji discusses generally available reports and studies relevant to the safety of *L. japonica* extract. Fuji cites a 1973 report by the Select Committee on GRAS Substances[(2)] regarding the safety of brown algae and red algae as food ingredients. The committee concluded that there was no substantive evidence or reason to suspect a significant risk to public health from use of these ingredients as spices, seasonings, and flavorings. Fuji notes that standard toxicity studies are not found in the literature because *L. japonica* has a history of consumption as food, but that some studies have been conducted to evaluate potential beneficial effects of *L. japonica*. In this regard, Fuji describes the results of human clinical and epidemiological studies and of in vitro and animal studies that have evaluated various effects of *L. japonica* and other *Laminaria* species or their extracts.

Fuji acknowledges that excessive iodine consumption can have adverse effects at high levels but notes that such effects appear to be reversible. Based on its estimate that intake of *L. japonica* extract at the 90th percentile would be approximately 150 mg/p/d, and its

specification that the iodine content of *L. japonica* extract is less than or equal to approximately 1600 milligrams iodine per kilogram of extract, Fuji estimates that intake of iodine from the consumption of *L. japonica* extract would be approximately 240 micrograms/p/d[(3)] and notes that this intake is several-fold less than the intake that has been associated with adverse effects of iodine. Fuji also notes that FDA has approved the addition of kelp to food as a source of iodine, provided that the maximum intake of the food as may be consumed during a period of one day does not result in daily ingestion of kelp so as to provide a total amount of iodine in excess of 225 micrograms (21 CFR 172.365).

As part of its notice, Fuji includes the report of a panel of individuals (Fuji's GRAS panel) who evaluated the data and information that are the basis for Fuji's GRAS determination. Fuji considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Fuji's GRAS panel evaluated dietary exposure, identity of kelp source material, method of production, and product specifications. Fuji's GRAS panel reviewed the reported history of use of *L. japonica* as a foodstuff in the United States and Japan, as well as data and information from human and in vitro studies. Fuji's GRAS panel concluded that *L. japonica* extract, meeting food grade specifications, is GRAS for its intended use.

Potential Labeling Issues

Section 403(a) of the Federal Food, Drug and Cosmetic Act (FFDCA) provides that a food shall be deemed to be misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FFDCA lays out the statutory framework for health claims. In describing information that the notifier relies on to conclude that *L. japonica* extract is GRAS under the conditions of its intended use, Fuji raises issues under these labeling provisions of the FFDCA. These issues include Fuji's description of animal studies suggesting that seaweeds and seaweed extracts may have beneficial effects. These issues are the purview of the Office of Nutritional Products, Labeling and Dietary Supplements (ONPLDS) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety neither consulted with ONPLDS on these labeling issues nor evaluated the information in Fuji's notice to determine whether it would support any claims made about *L. japonica* extract on the label or in labeling.

Use in Meat and Poultry Products

The intended use of *L. japonica* extract in products such as marinades, soups, gravies, and seasonings will result in the presence of *L. japonica* extract in meat and poultry products. Therefore, during its evaluation of GRN 000123, OFAS consulted with the Labeling and Consumer Protection Staff of the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA). Under the Federal Meat Inspection Act and the Poultry Products Inspection Act, FSIS is responsible for determining the efficacy and suitability of food ingredients in meat and poultry products as well as prescribing safe conditions of use. Suitability relates to the effectiveness of the ingredient in performing the intended purpose of use and the assurance that the conditions of use will not result in an adulterated product, or one that misleads consumers.

FSIS had no objection to the use of *L. japonica* extract (identified as such on labeling) as a flavor enhancer and flavoring agent in marinades for meat and poultry, meat and poultry

soups, gravies, and seasonings at a level not to exceed 0.08 percent by weight. FSIS notes that the addition of salt to the extract would require ingredient labeling.

Conclusions

Based on the information provided by Fuji, as well as the information in GRN 000123 and other information available to FDA, the agency has no questions at this time regarding Fuji's conclusion that *L. japonica* extract is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of *L. japonica* extract As always, it is the continuing responsibility of Fuji to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

cc: Dr. Robert Post, Director
Labeling and Consumer Protection Staff
Office of Policy, Program Development and Evaluation
Food Safety and Inspection Service
1400 Independence Ave., SW, Suite 602, Annex
Washington, DC 20250-3700

References

1. Pennington, J.A.T., S.A. Schoen, G.D. Salmon, B. Young, R.D. Johnson, and R.W. Marts, 1995. Composition of core foods in the U.S. food supply, 1982-1991. Journal of Food Composition and Analysis 8:171-217.

2. "Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickle, Silicon, Vanadium, and Zinc" (2002) Food and Nutrition Board, Institute of Medicine, National Academy of Sciences. Available online at http://www.nap.edu.

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(1)Based on Fuji's proposed conditions of use, FDA made an independent estimate that intake of *L. japonica* extract would be approximately 300 mg/p/d at the 90th percentile. FDA's estimate of exposure is based on the conservative assumptions that *L. japonica* extract is added at the maximum use level to all soups and marinades, and that all soups and marinades would contain the extract.

[(2)]Several years ago, FDA contracted with the Life Sciences Research Office (LSRO) of the Federation of American Societies for Experimental Biology as part of its comprehensive review of GRAS and prior sanctioned food ingredients. To aid in that review, LSRO established the Select Committee on GRAS Substances.

[(3)]Based on its independent estimate of intake of *L. japonica* extract, FDA estimated that the concomitant intake of iodine at the 90th percentile would be approximately 460 micrograms/p/d. FDA notes that the 90th percentile value for the usual intake of iodine from food (including added or naturally-occurring iodine) also is 460 micrograms/p/d, based in part on FDA's Total Diet Study (Ref. 1) as described by the National Academy of Sciences (Ref. 2). If iodine exposure from the use of *L. japonica* extract is conservatively assumed to be in addition to the background dietary intake of iodine, the resultant intake approaches, but does not exceed, the tolerable upper limit of intake of iodine for adults of 1100 micrograms/p/d as described by the National Academy of Sciences (Ref. 2). (By definition, the tolerable upper limit represents the highest level of daily intake of iodine that is likely to pose no risk of adverse health effects in almost all individuals.)

Page Last Updated: 04/17/2013
Note: If you need help accessing information in different file formats, see Instructions for Downloading Viewers and Players.

U.S. Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993
Ph. 1-888-INFO-FDA (1-888-463-6332)
Email FDA

AgriPrime Nematec® is a specific formulation derived from the Laminaria species of seaweed. It is a soluble liquid concentrate that is designed for foliar application.

APPLICATION RATES AND TIMINGS:

CROPS	Dose	N° of app.	Directions for use
Fruit crops, such as (but not limited to) Apples, Apricots, Avocados, Blueberries, Cherries, Citrus, Cranberries, Currants, Figs, Grapes (Table, Raisin & Juice), Grapes (Wine), Gooseberry, Kiwis , Olives, Pear, Peaches, Plums, Prunes, Nectarines, Strawberries	2.5L/Ha	4-5	Commence the application (foliar) when new flush of leaves and roots emerge in a growing season. Minimum retreatment interval should be 7 days. Increase the number of applications when stress level is high. Treat each flush of new growth in a growing season in fruit trees when the stress level is high.
Tree nuts such as (but not limited to) Almonds, Cashews, chestnuts, Hazelnuts, Pistachios, Pecans, walnuts			
Tropical/Sub Tropical Fruits (but not limited to) Avocados, Coffee, Durian, Mangos, Papaya, Pineapples, Pomegranate, Lychees, Bananas			
Vegetables (Fruiting) such as (but not limited to) Eggplant, Peppers, Fresh tomatoes, Processing tomatoes, Okra,	2.5L/Ha	4-5	Commence the application (foliar) when new flush of leaves and roots emerge in a growing season (50% emergence of seedlings). Minimum retreatment interval should be 7 days. Increase the number of applications when stress level is high.
Leafy vegetables such as (but not limited to) Lettuce, Celery, Spinach, Parsley, Radicchio			
Bulb vegetables such as (but not limited to) Garlic, leeks, Onions, Shallots,			
Cole crops such as (but not limited to) Broccoli, Brussels Sprouts, Cauliflower, Cabbage, Collards, Kale, Mustard greens			
Cucurbits such as (but not limited to) Cucumber, Cantaloupe, Squash, Pumpkin, Melons,			
Legumes and Pulses such as (but not limited to) Beans, Green Beans, Snap Beans, Lentils, Peas, Soybeans.			
Root, tuber and corm vegetables such as (but not limited to) Beets, Carrots, Potatoes, Sweet Potatoes, Ginger, Radish, Turnip, Yam			
Field crops such as (but not limited to) Corn (Grain, Feed & Forage); Cotton, Hops, Rice, Wheat, Barley, Oats, Oilseed rape (Canola), Sunflower, Sugar Beets, Sugar Cane			
Grasses such as (but not limited to) Alfalfa, Hay, Forage			
Green-house & shade-house crops			
Ornamentals such as (but not limited to) Bare root, container, bedding and flowering plants, flowering trees, woody ornamentals.			
Turf and lawns	2.5L/Ha	4-5	Application instructions as above. Do not water the grass surface for at least 10-12 hours following application. Use a sprayer with a pressure nozzle to ensure the spray applications to reach lower part of turf leaves.

APPLICATION:

Water volume for application: Recommended to apply 2.5L/Ha diluted in 250L of water for optimum results.

Mixing: Shake the product well before opening. Fill the sprayer tank with half the required volume of water. Begin agitation and slowly add the required amount of Ocean Green Nematec. Maintain agitation throughout the spraying process.

Application Recommendations: Care should be taken to thoroughly spray the dilute solutions of Ocean Green Nematec onto the leaves to a point of run-off. Avoid applying at peak daily temperature. For best results, apply early in the morning or late in evening.

Storage: Store above 5°C/41°F and away from direct sunlight.

Caution: Keep out of reach of children.

Garantia del Vendedor: La garantia del vendedor se limitarà a los términos dispuestos en la etiqueta y sujeto al mismo. Los resultados podrian variar. El comprador asume el riesgo de personas o propiedades surgidas en el uso o manejo de este producto y acepta el producto en esta condición.

5 390372 013100

BioAtlantis

Nature working Naturally™

AgriPrime Nematec®

0–0–3

A concentrated solution of seaweed extract from *Laminaria* species

Guaranteed minimum analysis:

Soluble Potash (K_2O): 3%
Derived from: Seaweed (*Laminaria* species)
(0.469 lbs minimum dry matter content per pint)

Net Content: 2.75 gallons / 10.41 litres
Net Weight: 28.2 lbs / 12.8 kg
Specific Gravity : 1.23

Manufactured by: BioAtlantis Ltd., Tralee, County Kerry, Ireland, Tel. + 353 (0)66 711 8477
www.bioatlantis.com
Product of Ireland

Ecolicitor® is a specific formulation derived from *Ascophyllum nodosum* seaweed. It is designed to enhance the following: seed emergence, root growth, shoot growth and lesion reduction.

DIRECTIONS FOR USE:

Ecolicitor® is a soluble liquid concentrate that is designed for foliar application and fertigation. Ensure maximum coverage to optimize results.

APPLICATION RATES AND TIMINGS:

CROPS	Foliar Application Timings				
	First (2.0 L/Ha)	Second (2.0 L/Ha)		Third (2.0 L/Ha)	Fourth (2.0 L/Ha)
Apples, Pears, Cherries, Apricots	Petal fall	Early fruit formation		Every 14 days until harvest	
Almonds, Pistachios, Walnuts & Avocado	Bloom / Onset of flowering	Initial fruit set		4 weeks after flowering, then every 14 days	
Bananas	Bud break	Bunch formation		Every 14 days until harvest	
Citrus & Berries	2 weeks pre-bloom	Petal fall		Repeat 2-4 weeks	
	First (2.5 L/Ha)	Second (2.5 L/Ha)	Third (2.5 L/Ha)	Fourth (1.5 L/Ha)	Fifth (1.5 L/Ha)
Grapevines	2.5-10 cm shoot growth (foliar and soil)	30 cm shoot growth (foliar and soil)		5-10 days pre-bloom	Bunch Formation and at anticipated stress
Aubergines, Peppers, Tomatoes, Beans, Peas, Lentils & Melons	1 week after transplanting	15 days after transplanting	30 days after transplanting	Repeat foliar treatments from first fruit set / first pod, every 14 days until harvest	
Cole crops (Cabbages) & leafy (Lettuce)	At first 3 leaf stage	7 days later	14 days later	30% of head (leaf) initiation	50% of final weight
Cucumbers, Squash, Pumpkins & Strawberries	At 4 leaf stage / prior to transplant	10-14 days after transplant	30 days after transplanting	First pre-bloom	Every 14 days through picking
Corn, Sweet Corn	Soil treatment at planting	At 2-6 leaf stage	15 days later	At 40-60 cm stage	
Root Crops (Carrots), Bulbs (Onions)	2-3 weeks after emergence	At root enlargement	15 days later	Every 14 days until harvest	
Rice	2-3 tillers	Panicle initiation	15 days later	Early flowering	2 weeks after flowering
Tuber crops (Potatoes, Beet, etc.)	At 50% emergence	7 days after first application	14 days after first application	At tuber set	10 - 14 days later
Winter Wheat, Spring Wheat, Oats	Soil treatment at planting	First node	15 days later	Flag leaf emergence	Before any anticipated stress
Fertigation	2 litre / 500 litre of water per hectare		1.5 litre / 500 litre of water per hectare, Every 14 days		
Greenhouse Crops & Ornamentals	1-2 litre / 500 litre of water per hectare		1.5 litre / 500 litre of water per hectare, Every 14 days or after every cut or new bud		
Sports Turf & Golf Courses	1.5-2 litre / 500 litre of water per hectare		1.5 litre / 500 litre of water per hectare, Every 14 days		

APPLICATION:

Water volumes for foliar applications: Recommended to apply 1.5 - 2.5 litre / 500 litre of water per Ha for optimum results.

Mixing: Shake the product well before opening. Fill the sprayer tank with half the required volume of water. Begin agitation and slowly add the required amount of **Ecolicitor®**. Maintain agitation throughout the spraying process. Diluted solutions of **Ecolicitor®** should be sprayed promptly.

Application Recommendations: Applications should not be made during extreme daytime temperatures or in extremely strong sunshine. For best results apply in cool conditions, preferably in early morning or in the evening. Application of an adjuvant is not required. Contact us for specific use recommendation.

STORAGE:

Store above 5°C and away from direct sunlight.

CAUTION:

Keep out of reach of children.

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CONCLUSION ON PESTICIDE PEER REVIEW

Conclusion on the peer review of the pesticide risk assessment of the active substance sea-algae extract[1]

European Food Safety Authority[2]

European Food Safety Authority (EFSA), Parma, Italy

SUMMARY

Sea-algae extract is one of the 295 substances of the fourth stage of the review programme covered by Commission Regulation (EC) No 2229/2004[3], as amended by Commission Regulation (EC) No 1095/2007[4].

Sea-algae extract was included in Annex I to Directive 91/414/EEC on 1 September 2009 pursuant to Article 24b of the Regulation (EC) No 2229/2004 (hereinafter referred to as 'the Regulation'), and has subsequently been deemed to be approved under Regulation (EC) No 1107/2009[5], in accordance with Commission Implementing Regulation (EU) No 540/2011[6], as amended by Commission Implementing Regulation (EU) No 541/2011[7]. In accordance with Article 25a of the Regulation, as amended by Commission Regulation (EU) No 114/2010[8], the European Food Safety Authority (EFSA) is required to deliver by 31 December 2012 its view on the draft review report submitted by the European Commission in accordance with Article 25(1) of the Regulation. This review report was established as a result of the initial evaluation provided by the designated rapporteur Member State in the Draft Assessment Report (DAR). The EFSA therefore organised a peer review of the DAR. The conclusions of the peer review are set out in this report.

Italy being the designated rapporteur Member State submitted the DAR on sea-algae extract in accordance with the provisions of Article 22(1) of the Regulation, which was received by the EFSA on 28 November 2007. The peer review was initiated on 18 June 2008 by dispatching the DAR to the notifier the Seaweed Task Force and on 24 February 2011 to the Member States for consultation and comments. Following consideration of the comments received on the DAR, it was concluded that there was no need to conduct an expert consultation and EFSA should deliver its conclusions on sea-algae extract.

The conclusions laid down in this report were reached on the basis of the evaluation of the representative uses of sea-algae extract as a plant growth regulator on beans, as proposed by the notifier. Full details of the representative uses can be found in Appendix A to this report.

[1] On request from the European Commission, Question No EFSA-Q-2009-00289, issued on 6 December 2011.
[2] Correspondence: pesticides.peerreview@efsa.europa.eu
[3] OJ L 379, 24.12.2004, p.13
[4] OJ L 246, 21.9.2007, p.19
[5] OJ L 309, 24.11.2009, p.1
[6] OJ L 153, 11.6.2011, p.1
[7] OJ L 153, 11.6.2011, p.187
[8] OJ L 37, 10.2.2010, p.12

Suggested citation: European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance sea-algae extract. EFSA Journal 2012;10(1):2492. [42 pp.] doi:10.2903/j.efsa.2012.2492. Available online: www.efsa.europa.eu/efsajournal

In the area of identity, physical/chemical/technical properties and methods of analysis the following Annex II data gaps were identified: melting point, boiling point, temperature of decomposition, vapour pressure, Henry's law constant, spectra, solubility in water, solubility in organic solvents, octanol-water partition co-efficient, hydrolysis, photolysis, quantum yield and dissociation constant. For the formulations low temperature stability, dilution stability before and after accelerated storage, and shelf-life studies were identified as data gaps.

Data gaps were identified in the mammalian toxicology section regarding information on the uses allowing to waive toxicological studies for the species *Macrocystis integrifolia*, and for medical data on the three species *Ascophyllum nodosum, Laminaria digitata* and *Macrocystis integrifolia*.

No areas of concern or data gaps were identified in the residue section.

No areas of concern or data gaps were identified in the environmental fate and behaviour section.

The risk to birds and mammals and to aquatic organisms was assessed as low. However, a data gap was identified for further data and risk assessment for algae from an additional taxonomic group and for aquatic plants. Moreover, it was noted that the composition of the batches of the formulations used in the aquatic tests did not comply with the representative batches. A data gap was also identified for a risk assessment for honeybees referring to one of the representative uses where exposure of bees could occur. Further data gaps were identified for non-target arthropods, earthworms, soil macro- and micro-organisms and terrestrial non-target plants. The extent of the risk to these non-target organisms could not be assessed.

KEY WORDS

Sea-algae extract, peer review, risk assessment, pesticide, plant growth regulator

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BACKGROUND

Sea-algae extract is one of the 295 substances of the fourth stage of the review programme covered by Commission Regulation (EC) No 2229/2004[9], as amended by Commission Regulation (EC) No 1095/2007[10].

Sea-algae extract was included in Annex I to Directive 91/414/EEC on 1 September 2009 pursuant to Article 24b of the Regulation (EC) No 2229/2004 (hereinafter referred to as 'the Regulation'), and has subsequently been deemed to be approved under Regulation (EC) No 1107/2009[11], in accordance with Commission Implementing Regulation (EU) No 540/2011[12], as amended by Commission Implementing Regulation (EU) No 541/2011[13]. In accordance with Article 25a of the Regulation, as amended by Commission Regulation (EU) No 114/2010[14] the European Food Safety Authority (EFSA) is required to deliver by 31 December 2012 its view on the draft review report submitted by the European Commission in accordance with Article 25(1) of the Regulation (European Commission, 2008). This review report was established as a result of the initial evaluation provided by the designated rapporteur Member State in the Draft Assessment Report (DAR). The EFSA therefore organised a peer review of the DAR. The conclusions of the peer review are set out in this report.

Italy being the designated rapporteur Member State submitted the DAR on sea-algae extract in accordance with the provisions of Article 22(1) of the Regulation, which was received by the EFSA on 28 November 2007 (Italy, 2007). The peer review was initiated on 18 June 2008 by dispatching the DAR to the notifier the Seaweed Task Force and on 24 February 2011 to the Member States for consultation and comments. In addition, the EFSA conducted a public consultation on the DAR. The comments received were collated by the EFSA and forwarded to the RMS for compilation and evaluation in the format of a Reporting Table. The notifier was invited to respond to the comments in column 3 of the Reporting Table. The comments and the notifier's response were evaluated by the RMS in column 3 of the Reporting Table.

The scope of the peer review was considered in a telephone conference between the EFSA, the RMS, and the European Commission on 20 June 2011. On the basis of the comments received and the RMS' evaluation thereof it was concluded that there was no need to conduct an expert consultation.

The outcome of the telephone conference, together with EFSA's further consideration of the comments is reflected in the conclusions set out in column 4 of the Reporting Table. All points that were identified as unresolved at the end of the comment evaluation phase and which required further consideration, and the additional information to be submitted by the notifier, were compiled by the EFSA in the format of an Evaluation Table.

The conclusions arising from the consideration by the EFSA, and as appropriate by the RMS, of the points identified in the Evaluation Table, were reported in the final column of the Evaluation Table.

A final consultation on the conclusions arising from the peer review of the risk assessment took place with Member States via a written procedure in November 2011.

This conclusion report summarises the outcome of the peer review of the risk assessment on the active substance and the representative formulation evaluated on the basis of the representative uses as a plant growth regulator on beans, as proposed by the notifier. A list of the relevant end points for the active substance as well as the formulation is provided in Appendix A. In addition, a key supporting document to this conclusion is the Peer Review Report, which is a compilation of the documentation developed to evaluate and address all issues raised in the peer review, from the initial commenting

[9] OJ L 379, 24.12.2004, p.13
[10] OJ L 246, 21.9.2007, p.19
[11] OJ L 309, 24.11.2009, p.1
[12] OJ L 153, 11.6.2011, p.1
[13] OJ L 153, 11.6.2011, p.187
[14] OJ L 37, 10.2.2010, p.12

phase to the conclusion. The Peer Review Report (EFSA, 2011) comprises the following documents, in which all views expressed during the course of the peer review, including minority views, can be found:

- the comments received on the DAR,
- the Reporting Table (21 June 2011),
- the Evaluation Table (1 December 2011),
- the comments received on the assessment of the points of clarification,
- the comments received on the draft EFSA conclusion.

Given the importance of the DAR including its addendum (compiled version of September 2011 containing all individually submitted addenda (Italy, 2011)) and the Peer Review Report, both documents are considered respectively as background documents A and B to this conclusion.

THE ACTIVE SUBSTANCE AND THE FORMULATED PRODUCT

The materials being considered are sea-algae extracts; there are no IUPAC or ISO names for these materials. These are aqueous extracts of one or more of the species *Ascophyllum nodosum*, *Laminaria digitata* and *Macrocystis integrifolia* of the orders *Fucales* and *Laminariales* of the class *Phaeophyceae* (brown seaweeds). The sea-algae used as the starting material is of food grade quality.

The representative formulated products for the evaluation were 'Kelpgrow', 'Agrocean base', 'Stimplex', 'Althia' and 'Algaegreen'.

The representative uses evaluated are outdoor spray applications to beans as a plant growth regulator. Full details of the representative uses can be found in the list of end points in Appendix A.

CONCLUSIONS OF THE EVALUATION

1. Identity, physical/chemical/technical properties and methods of analysis

The following guidance document was followed in the production of this conclusion: SANCO/3030/99 rev.4 (European Commission, 2000).

Acceptable marker compound specifications were provided for all of the extracts. It is noted that no information was given on the level of microbial contamination and the mechanism for the control of such contamination, or its possible increase on storage.

The following Annex II data points were not sufficiently addressed: melting point, boiling point, temperature of decomposition, vapour pressure, Henry's law constant, spectra, solubility in water, solubility in organic solvents, octanol-water partition co-efficient, hydrolysis, photolysis, quantum yield and dissociation constant. These can be addressed with a reasoned case.

The main data regarding the identity of the extracts and their physical and chemical properties are given in Appendix A.

For all plant protection products the following data gaps were identified: low temperature stability, dilution stability before and after accelerated storage, and shelf-life studies.

Methods of analysis for residues are not required due to the nature of these extracts. A method of analysis for body fluids and tissues is not required as the extracts are not classified as toxic or very toxic.

2. Mammalian toxicity

Sea-algae extracts do not have a toxic mode of action and do not present a toxicological concern by themselves. As sea-algae are harvested in a variable natural environment where contaminants of toxicological concern such as heavy metals, toxins produced as secondary metabolites from blue-green algae or cyanobacteria, and pathogens are potentially present, the toxicological assessment assumes that the manufacturing process ensures the production of a food grade quality of the extract.

Sea-algae extracts are also used as herbal remedies, however no information has been submitted on their (beneficial or adverse) effects and therefore a data gap was identified for medical data.

Based on the nature of sea-algae extract from the species *Ascophyllum nodosum* and *Laminaria digitata* used as seaweed meal in animal and human nutrition, and in health food tablets or gelatine capsules, all toxicological data requirements are waived for these two species. Toxicological reference values are not required and no quantitative risk assessment for operators, workers or bystanders was conducted considering the risk, if any, to be negligible. A data gap has been identified for information on the uses related to the species *Macrocystis integrifolia* that would allow to waive toxicological

information also on this species. However, it is expected that the species *Macrocystis integrifolia* would also be used in human nutrition.

3. Residues

To assess the consumer risk from the representative uses of sea-algae extract the assessment was conducted by comparison of the exposure arising from the use as a plant protection product with the exposure arising from consumption of the plant itself. The assessment presumes that the sea-algae extract used will be free of potentially harmful contaminants such as marine toxins, heavy metals or pathogens.

Consumption data of aquatic plants for EU countries can be extracted from the respective WHO cluster diets B, E, F and D (WHO, 2006), and range from 0.1 to 30.8 g/person/day. Having regard to the single application and the representative dose rate, it is considered unlikely that any pre-existing daily dietary exposure of humans to aquatic plants would be significantly increased by the use of sea-algae extract as a plant protection product.

No areas of concern or data gaps are identified. No MRL is proposed; sea-algae extract could be considered as a candidate for Annex IV of Commission Regulation (EC) No 396/2005[15].

4. Environmental fate and behaviour

The sea-algae extract products are all aqueous extracts (cell contents) of one or more of the species *Ascophyllum nodosum*, *Laminaria digitata* and *Macrocystis integrifolia*. No information or experimental data on these algal products were submitted in the dossier. However, it is considered that algae and algal products, used as soil supplements, are readily transformed to elements naturally present in the environment. Therefore, when the formulations containing sea-algae extracts are applied to bean plants, they are expected to degrade resulting in a low potential for longer term impact on the environment. However, as the exposure of soil and natural surface water systems might be expected to be low but cannot be completely excluded, initial PEC for the product in soil and surface water via drift have been estimated and were included in an Addendum (Italy, 2011). Although the method of calculation for PECsw is not completely clear, the EFSA considers that the available values are conservative and can be considered acceptable. These PEC are included in appendix A.

5. Ecotoxicology

For the environmental risk assessments the following documents were considered: European Commission 2002a and 2002b, and EFSA, 2009.

No toxicity studies were available for **birds** therefore no quantitative risk assessments were performed. Considering other available information, such as the fact that seaweed products are routinely used in poultry-feeding, and the available toxicological end points for mammals, it was concluded that the risk to birds from the use of sea-algae extract as a pesticide, based on the representative uses, is low. This was further supported by the available risk assessment for wild mammals that indicated low risk to **non-target terrestrial vertebrates** other than birds.

Risk assessments for **aquatic organisms,** based on the available acute data for fish, daphnia and algae with the formulations and considering a spray drift exposure of the aquatic environment, resulted in a low risk. It was noted that the composition of the batches of formulations used in the aquatic tests did not comply with the representative batches. No data for long-term toxicity were available. However, considering the nature and the composition of the products, the available toxicity data and the representative uses of these products, no assessments were considered to be necessary for long-term scale. However, a data gap has been identified for further data and risk assessment for algae from an additional taxonomic group, and for aquatic plants considering that sea-algae extracts are plant growth regulators.

[15] OJ L 70, 16.3.2005, p. 16

No toxicity data or risk assessments for **honeybees** were available. However, based on the representative uses, four out of the five formulations are applied only when attractive crops or flowers are not present in the field. Therefore the exposure of bees was considered to be negligible for these uses. However, this is not the case for the representative use with the formulation 'Agrocean Base' that can be applied also in later growth stages when the presence of other attractive crops or flowering weeds cannot be excluded, therefore a data gap has been identified for a risk assessment for honeybees for the case(s) when bees can be exposed.

No reliable data or risk assessments were available for **non-target arthropods**, **earthworms**, **soil macro- and micro- organisms** or for **terrestrial non-target plants**. Considering the facts that no data are available, the composition of the formulations contains several compounds, some of which are plant hormones, and that the mode of action was not fully clarified, the extent of the risk to these non-target organisms could not be assessed. Therefore relevant data gaps were identified for the assessments for these issues.

The risk to **biological methods for sewage treatments** was considered as low.

6. Overview of the risk assessment of compounds listed in residue definitions triggering assessment of effects data for the environmental compartments

6.1. Soil

Compound (name and/or code)	Persistence	Ecotoxicology
Sea-algae extract	No data, not required	No data were available. Data gap.

6.2. Ground water

Compound (name and/or code)	Mobility in soil	>0.1 µg/L 1m depth for the representative uses (at least one FOCUS scenario or relevant lysimeter)	Pesticidal activity	Toxicological relevance	Ecotoxicological activity
Sea-algae extract	No data, not required	No data, not required	Not applicable	No	The risk to aquatic organisms was assessed as low. Data gap was identified for further data and assessments for algae from an additional taxonomic group, and for aquatic plants.

6.3. Surface water and sediment

Compound (name and/or code)	Ecotoxicology
Sea-algae extract	The risk to aquatic organisms was assessed as low. Data gap was identified for further data and assessments for algae from an additional taxonomic group, and for aquatic plants.

6.4. Air

Compound (name and/or code)	Toxicology
Sea-algae extract	No data - not required

7. List of studies to be generated, still ongoing or available but not peer reviewed

This is a complete list of the data gaps identified during the peer review process, including those areas where a study may have been made available during the peer review process but not considered for procedural reasons (without prejudice to the provisions of Article 7 of Directive 91/414/EEC concerning information on potentially harmful effects).

- Melting point, boiling point, temperature of decomposition, vapour pressure, Henry's law constant, spectra, solubility in water, solubility in organic solvents, octanol-water partition co-efficient, hydrolysis, photolysis, quantum yield and dissociation constant. These can be addressed with a reasoned case (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 1)

- For all formulations low temperature stability (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 1)

- For all formulations dilution stability before and after accelerated storage (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 1)

- For all formulations a shelf-life study (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 1)

- Medical data on sea-algae extracts from the three species *Ascophyllum nodosum*, *Laminaria digitata* and *Macrocystis integrifolia* (relevant for all representative uses evaluated; submission date proposed by the notifier: some data were provided in Reporting Table point 2(6), however according to Commission Regulation (EC) No 1095/2007 new information cannot be considered in the peer review; see section 2)

- Information on the uses of the species *Macrocystis integrifolia* that would allow to waive toxicological information (relevant for the representative use with the formulation "Kelpgrow"; submission date proposed by the notifier: indications were provided in Reporting Table point 2(7), however according to Commission Regulation (EC) No 1095/2007 new information cannot be considered in the peer review; see section 2)

- Additional data and risk assessments for algae from an other taxonomic group and for aquatic plants (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 5)

- Risk assessment for honeybees for the case(s) when bees can be exposed (relevant for the representative use with the formulation 'Agrocean Base'; submission date proposed by the notifier: unknown; see section 5)

- Risk assessment for non-target arthropods (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 5)

- Risk assessment for non-target soil organisms (such as earthworms, soil macro- and micro-organisms) (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 5)

- Risk assessment for terrestrial non-target plants (relevant for all representative uses evaluated; submission date proposed by the notifier: unknown; see section 5)

8. Particular conditions proposed to be taken into account to manage the risk(s) identified

- The toxicological assessment assumes that the manufacturing process ensures a food grade quality of sea-algae extracts.

9. Concerns

9.1. Issues that could not be finalised

An issue is listed as an issue that could not be finalised where there is not enough information available to perform an assessment, even at the lowest tier level, for the representative uses in line with the Uniform Principles of Annex VI to Directive 91/414/EEC and where the issue is of such importance that it could, when finalised, become a concern (which would also be listed as a critical area of concern if it is of relevance to all representative uses).

1. The extent of the risk to honeybees for the representative use with the formulation 'Agrocean Base' when the treated area is potentially attractive to bees

2. The extent of the risk to non-target arthropods

3. The extent of the risk to non-target soil organisms

4. The extent of the risk to non-target terrestrial plants

9.2. Critical areas of concern

An issue is listed as a critical area of concern where there is enough information available to perform an assessment for the representative uses in line with the Uniform Principles of Annex VI to Directive 91/414/EEC, and where this assessment does not permit to conclude that for at least one of the representative uses it may be expected that a plant protection product containing the active substance will not have any harmful effect on human or animal health or on groundwater or any unacceptable influence on the environment.

An issue is also listed as a critical area of concern where the assessment at a higher tier level could not be finalised due to a lack of information, and where the assessment performed at the lower tier level does not permit to conclude that for at least one of the representative uses it may be expected that a plant protection product containing the active substance will not have any harmful effect on human or animal health or on groundwater or any unacceptable influence on the environment.

None.

9.3. Overview of the concerns identified for each representative use considered

(If a particular condition proposed to be taken into account to manage an identified risk, as listed in section 8, has been evaluated as being effective, then 'risk identified' is not indicated in this table.)

Representative use		**Outdoor spray applications to beans as a plant growth regulator**	
		Max. application rate of 2 L formulated product/ha ('Kelpgrow', 'Stimplex', 'Althia', 'Algaegreen')	Max. application rate of 2.5 L formulated product/ha ('Agrocean Base')
Operator risk	Risk identified		
	Assessment not finalised		
Worker risk	Risk identified		
	Assessment not finalised		
Bystander risk	Risk identified		
	Assessment not finalised		
Consumer risk	Risk identified		
	Assessment not finalised		
Risk to wild non target terrestrial vertebrates	Risk identified		
	Assessment not finalised		
Risk to wild non target terrestrial organisms other than vertebrates	Risk identified		
	Assessment not finalised	X[2,3,4]	X[1,2,3,4]
Risk to aquatic organisms	Risk identified		
	Assessment not finalised		
Groundwater exposure active substance	Legal parametric value breached		
	Assessment not finalised		
Groundwater exposure metabolites	Legal parametric value breached		
	Parametric value of 10µg/L[(a)] breached		
	Assessment not finalised		
Comments/Remarks			

The superscript numbers in this table relate to the numbered points indicated in sections 9.1 and 9.2. Where there is no superscript number see sections 2 to 6 for further information.

(a): Value for non relevant metabolites prescribed in SANCO/221/2000-rev 10-final, European Commission, 2003

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APPENDICES

APPENDIX A – LIST OF END POINTS FOR THE ACTIVE SUBSTANCE AND THE REPRESENTATIVE FORMULATION

Identity, Physical and Chemical Properties, Details of Uses, Further Information, Methods of Analysis

Identity, Physical and Chemical Properties, Details of Uses, Further Information

Active substance (ISO Common Name) ‡	Sea-algae extract (No ISO common name available)
Function (*e.g.* fungicide)	Plant growth regulator
Rappporteur Member State	Italy

Identity (Annex IIA, point 1)

Chemical name (IUPAC) ‡	Not applicable
Chemical name (CA) ‡	Not applicable
CIPAC No ‡	Not applicable
CAS No ‡	Not applicable
EC No (EINECS or ELINCS) ‡	Not applicable
FAO Specification (including year of publication) ‡	Not applicable
Minimum purity of the active substance as manufactured (g/kg) ‡	See Appendix B – Detailed specification of the marker compounds in the formulations
Identity of relevant impurities (of toxicological, environmental and/or other significance) in the active substance as manufactured (g/kg)	None.
Molecular formula‡	Not applicable
Molecular mass‡	Not applicable
Structural formula‡	Not applicable

Physical and chemical properties (Annex IIA, point 2)

Melting point (state purity) ‡	Data gap
Boiling point (state purity) ‡	Data gap
Temperature of decomposition (state purity)	Data gap
Appearance (state purity) ‡	From solution to cream, colour from bright yellow to dark brown.
Vapour pressure (state temperature, state purity) ‡	Data gap
Henry's law constant (Pa m^3 mol^{-1}) ‡	Data gap
Solubility in water (g/l or mg/l, state temperature, state purity and pH) ‡	Data gap
Solubility in organic solvents (in g/l or mg/l, state temperature, state purity and pH) ‡	Data gap
pH	From 2.61 to 7.86
Kinematic viscosity	From 1.33 to 300-500 mm^2/s
Relative density (state purity)	From 1.032 to 1.109
Surface tension ‡	From 23.05 to 70.5 mN/m
Partition co-efficient (log P_{OW}) (state temperature, pH and purity) ‡	Data gap
Dissociation constant (state purity) ‡	Data gap
Acidity / Alkalinity	From 0.97% H_2SO_4 to 0.1% NaOH
Persistent foaming	No foam
Stability after storage for 14 days at 54° C	The products are stable
Stability after storage for other periods and/or temperatures	After accelerate storage stability test it can be concluded that the storage stability for other periods and/or temperatures is not required since the sea-algae extract is not heat sensitive.
UV/VIS absorption (max.) incl. ε (state purity, pH)‡	Data gap
Flammability ‡ (state purity)	Acceptable case provided
Explosive properties ‡ (state purity)	Acceptable case provided
Oxidising properties ‡ (state purity)	Acceptable case provided

Summary of representative uses evaluated (*sea-algae extract*)

Crop and/or situation (a)	Country	Product name (sponsor)	F G or I (b)	Pests or Group of pests controlled (c)	Formulation		Application				Application rate per treatment			PHI (days) (l)	Remarks: (m)
					Type (d-f)	Conc. of a.s. (i)	method kind (f-h)	growth stage & season (j)	number min max (k)	interval between applications (min)	kg a.s./hL min max	water (L/ha) min max	kg a.s./ha min max		
BEAN	FRANCE	KELPGROW (Asfaleia)	F	-	SL	--	spray	Growth stage 5 BBCH	1	-	-	-	2 litres of formulated product	Not necessary	-
BEAN	FRANCE	AGROCEAN BASE (Agrimer)	F	-	SL	--	spray	At any stage; avoid the blossoming stage of the culture	1	-	-	-	2 - 2.5 litres of formulated product	Not necessary	-
BEAN	FRANCE	STIMPLEX (Acadian)	F	-	SL	--	spray	Growth stage 5 BBCH	1	-	-	-	2 litres of formulated product	Not necessary	-
BEAN	FRANCE	ALTHIA (Goemar)	F	-	SL	--	spray	Growth stage 5 BBCH	1	-	-	-	2 litres of formulated product	Not necessary	-
BEAN	FRANCE	ALGAEGREEN (OGT)	F	-	SL	--	spray	Growth stage 5 BBCH	1	-	-	-	2 litres of formulated product	Not necessary	-

Remarks:

(a) For crops, Codex (or other, *e.g.* EU) classifications should be used; where relevant, the use situation should be described (*e.g.* fumigation of a structure)
(b) Outdoor or field use (F), glasshouse application (G) or indoor application (I)
(c) *e.g.* biting and sucking insects, soil borne insects, foliar fungi, weeds
(d) *e.g.* wettable powder (WP),emulsifiable concentrate (EC), granule (GR)
(e) GCPF Codes - GIFAP Technical Monograph No. 2, 1989
(f) All abbreviations must be explained
(g) Method, *e.g.* high volume spraying, low volume spraying, spreading, dusting, drench
(h) Kind, *e.g.* overall, broadcast, aerial spraying, row, individual plant, between the plants - type of equipment used must be indicated
(i) g/kg or g/l
(j) Growth stage at last treatment (BBCH Monograph, Growth Stages of Plants, 1997, Blackwell, ISBN 3-8263-3152-4), including where relevant, information on season at time of application
(k) The minimum and maximum number of applications possible under practical conditions of use must be provided
(l) PHI – minimum pre-harvest interval
(m) Remarks may include: Extent of use/ economic importance/restrictions

Methods of Analysis

Analytical methods for the active substance (Annex IIA, point 4.1)

The active substance can not be identified but three markers common to all extracts have been selected.

Technical a.s. (principle of method)	Not applicable
Impurities in technical a.s. (principle of method)	Not applicable
Plant protection product (principle of method)	Three markers have been identified: **Mannitol:** anion exchange chromatography coupled with the high sensitive pulsed amperometric detection (HPAE-PAD). **Fucose containing polymers (fucoidans):** Gibbons method (M.N. Gibbons – The determination of methylpentoses. Analyst, 1955, 80: 267-276) after precipitation by ethanol and re-dissolution by 30g/L $CaCl_2$ HCl 0.5 M solution. **Alginic acids and alginates:** metahydroxidiphenyl method with precipitation by 30 g/L $CaCl_2$ solution in ethanol and further dilution in sodium tetraborate solution.

Analytical methods for residues (Annex IIA, point 4.2)

The extracts are used as animal and/or human feed or herbal remedies and therefore there is no additional risk to consumers from any residues that may possibly occur as a result of the use as a plant protection product Therefore, no residue data requirements need to be fulfilled and no residue method is required.

Residue definitions for monitoring purposes

Food/feed of plant origin (principle of method and LOQ for methods for monitoring purposes)	Not required
Food/feed of animal origin (principle of method and LOQ for methods for monitoring purposes)	Not required
Soil (principle of method and LOQ)	Not required
Water (principle of method and LOQ)	Not required
Air (principle of method and LOQ)	Not required
Body fluids and tissues (principle of method and LOQ)	Not required

Monitoring/Enforcement methods

Food/feed of plant origin (analytical technique and LOQ for methods for monitoring purposes)	Not required
Food/feed of animal origin (analytical technique and LOQ for methods for monitoring purposes)	Not required

Soil (analytical technique and LOQ)	Not required
Water (analytical technique and LOQ)	Not required
Air (analytical technique and LOQ)	Not required
Body fluids and tissues (analytical technique and LOQ)	Not required

Classification and proposed labelling with regard to physical and chemical data (Annex IIA, point 10)

	RMS/peer review proposal
Active substance	None

Impact on Human and Animal Health

Sea-algae extracts are all aqueous extracts (cell contents) of one or more of the species *Ascophyllum nodosum*, *Laminaria digitata* and *Macrocystis integrifolia* of the Orders *Fucales* and *Laminariales* of the Class *Phaeophyceae* (Brown Seaweeds).

These species have a non-toxic mode of action and are non-toxic by themselves; they are used as animal and/or human feed or herbal remedies and therefore there is no additional risk to consumers from any residues that may possibly occur as a result of the use as a plant protection product. It is concluded, therefore, that toxicological data requirements do not need to be fulfilled.

Absorption, distribution, excretion and metabolism (toxicokinetics) (Annex IIA, point 5.1)

Rate and extent of oral absorption ‡	No data - not required
Distribution ‡	No data - not required
Potential for accumulation ‡	No data - not required
Rate and extent of excretion ‡	No data - not required
Metabolism in animals ‡	No data - not required
Toxicologically relevant compounds ‡ (animals and plants)	No data - not required
Toxicologically relevant compounds ‡ (environment)	No data - not required

Acute toxicity (Annex IIA, point 5.2)

Rat LD_{50} oral ‡	> 2000 mg/kg bw	
Rat LD_{50} dermal ‡	> 2000 mg/kg bw	
Rat LC_{50} inhalation ‡	No data - not required	
Skin irritation ‡	Non-irritant	
Eye irritation ‡	Non-irritant	
Skin sensitisation ‡	Non-sensitising (M & K)	

Short term toxicity (Annex IIA, point 5.3)

Target / critical effect ‡	No data - not required	
Relevant oral NOAEL ‡	No data - not required	
Relevant dermal NOAEL ‡	No data - not required	
Relevant inhalation NOAEL ‡	No data - not required	

Genotoxicity ‡ (Annex IIA, point 5.4)

No data - not required	

Long term toxicity and carcinogenicity (Annex IIA, point 5.5)

Target/critical effect ‡	No data - not required	
Relevant NOAEL ‡	No data - not required	
Carcinogenicity ‡	No data - not required	

Reproductive toxicity (Annex IIA, point 5.6)

Reproduction toxicity

Reproduction target / critical effect ‡	No data - not required	
Relevant parental NOAEL ‡	No data - not required	
Relevant reproductive NOAEL ‡	No data - not required	
Relevant offspring NOAEL ‡	No data - not required	

Developmental toxicity

Developmental target / critical effect ‡	No data - not required	
Relevant maternal NOAEL ‡	No data - not required	
Relevant developmental NOAEL ‡	No data - not required	

Neurotoxicity (Annex IIA, point 5.7)

Acute neurotoxicity ‡	No data - not required	
Repeated neurotoxicity ‡	No data - not required	
Delayed neurotoxicity ‡	No data - not required	

Other toxicological studies (Annex IIA, point 5.8)

Mechanism studies ‡	No data
Studies performed on metabolites or impurities ‡	No data

Medical data ‡ (Annex IIA, point 5.9)

No data, data required.

Summary (Annex IIA, point 5.10)	Value	Study	Safety factor
ADI ‡	No data - not required	-	-
AOEL ‡	No data - not required	-	-
ARfD ‡	No data - not required	-	-

Dermal absorption ‡ (Annex IIIA, point 7.3)

Formulations (Kelpgrow, Althia, Agrocean Base, Stimplex, Algaegreen)	No data - not necessary

Exposure scenarios (Annex IIIA, point 7.2)

Operator	No exposure assessment was deemed necessary, as the substance does not present a toxicological concern. Exposure to consumers already exists, as sea-algae extracts are food-grade.
Workers	No exposure assessment was deemed necessary, as the substance does not present a toxicological concern. Exposure to consumers already exists, as sea-algae extracts are food-grade.
Bystanders	No exposure assessment was deemed necessary, as the substance does not present a toxicological concern. Exposure to consumers already exists, as sea-algae extracts are food-grade.

Classification and proposed labelling with regard to toxicological data (Annex IIA, point 10)

	RMS/peer review proposal
Sea algae extracts	No classification required.

Residues

The extracts are used as animal and/or human feed or herbal remedies and therefore there is no additional risk to consumers from any residues that may possibly occur as a result of the use as a plant protection product is expected. Therefore, the definition of residues is not requested and no ADI is proposed.

Metabolism in plants (Annex IIA, point 6.1 and 6.7, Annex IIIA, point 8.1 and 8.6)

Plant groups covered	Not required
Rotational crops	Not required
Metabolism in rotational crops similar to metabolism in primary crops?	Not required
Processed commodities	Not required
Residue pattern in processed commodities similar to residue pattern in raw commodities?	Not required
Plant residue definition for monitoring	Not required
Plant residue definition for risk assessment	Not required
Conversion factor (monitoring to risk assessment)	Not required

Metabolism in livestock (Annex IIA, point 6.2 and 6.7, Annex IIIA, point 8.1 and 8.6)

Animals covered	Not required
Time needed to reach a plateau concentration in milk and eggs	Not required
Animal residue definition for monitoring	Not required
Animal residue definition for risk assessment	Not required
Conversion factor (monitoring to risk assessment)	Not required
Metabolism in rat and ruminant similar (yes/no)	-
Fat soluble residue: (yes/no)	-

Residues in succeeding crops (Annex IIA, point 6.6, Annex IIIA, point 8.5)

-

Stability of residues (Annex IIA, point 6 introduction, Annex IIIA, point 8 introduction)

-

Residues from livestock feeding studies (Annex IIA, point 6.4, Annex IIIA, point 8.3)

	Ruminant	Poultry	Pig
	Conditions of requirement of feeding studies		
Expected intakes by livestock ≥ 0.1 mg/kg diet (dry weight basis) (yes/no - If yes, specify the level)			
Potential for accumulation (yes/no):			
Metabolism studies indicate potential level of residues ≥ 0.01 mg/kg in edible tissues (yes/no)			
	Feeding studies (Specify the feeding rate in cattle and poultry studies considered as relevant) Residue levels in matrices : Mean (max) mg/kg		
Muscle			
Liver			
Kidney			
Fat			
Milk			
Eggs			

Summary of residues data according to the representative uses on raw agricultural commodities and feedingstuffs (Annex IIA, point 6.3, Annex IIIA, point 8.2)

Crop	Northern or Mediterranean Region, field or glasshouse, and any other useful information	Trials results relevant to the representative uses (a)	Recommendation/comments	MRL estimated from trials according to the representative use	HR (c)	STMR (b)

(a) Numbers of trials in which particular residue levels were reported *e.g.* 3 x <0.01, 1 x 0.01, 6 x 0.02, 1 x 0.04, 1 x 0.08, 2 x 0.1, 2 x 0.15, 1 x 0.17
(b) Supervised Trials Median Residue *i.e.* the median residue level estimated on the basis of supervised trials relating to the representative use
(c) Highest residue

Consumer risk assessment (Annex IIA, point 6.9, Annex IIIA, point 8.8)

ADI	Not required
TMDI (according to WHO European Diet) (% ADI)	Not required
TMDI (% ADI) according to national (to be specified) diets	
IEDI (WHO European Diet) (% ADI)	
NEDI (specify diet) (% ADI)	Not required
Factors included in IEDI and NEDI	Not applicable
ARfD	Not required
IESTI (% ARfD)	
NESTI (% ARfD) according to national (to be specified) large portion consumption data	
Acute exposure (% ARfD)	Not applicable

Processing factors (Annex IIA, point 6.5, Annex IIIA, point 8.4)

Crop/process/processed product	Number of studies	Processing factors		Amount transferred (%)
		Transfer factor	Yield factor	

Proposed MRLs (Annex IIA, point 6.7, Annex IIIA, point 8.6)

Crop or Crop Group	**Proposed MRLs**
Beans	No MRL proposed. Candidate for Annex IV of Commission Regulation (EC) No 396/2005

Fate and Behaviour in the Environment

Route of degradation (aerobic) in soil (Annex IIA, point 7.1.1.1.1)

Mineralization after 100 days ‡	No data submitted
Non-extractable residues after 100 days ‡	No data submitted
Metabolites requiring further consideration ‡ - name and/or code, % of applied (range and maximum)	No data submitted

Route of degradation in soil - Supplemental studies (Annex IIA, point 7.1.1.1.2)

Anaerobic degradation ‡

Mineralization after 100 days	No data submitted
Non-extractable residues after 100 days	No data submitted
Metabolites that may require further consideration for risk assessment - name and/or code, % of applied (range and maximum)	No data submitted

Soil photolysis ‡

Metabolites that may require further consideration for risk assessment - name and/or code, % of applied (range and maximum)	No data submitted

Rate of degradation in soil (Annex IIA, point 7.1.1.2, Annex IIIA, point 9.1.1)

Laboratory studies:

Parent	Aerobic conditions: no data submitted

Field studies ‡

Parent	Aerobic conditions: no data submitted

Soil accumulation and plateau concentration ‡	No data submitted

Laboratory studies ‡

Parent	Anaerobic conditions: no data submitted

Soil adsorption/desorption (Annex IIA, point 7.1.2)

Parent ‡ no data submitted

Mobility in soil (Annex IIA, point 7.1.3, Annex IIIA, point 9.1.2)

Column leaching ‡	No data submitted
Aged residues leaching ‡	No data submitted
Lysimeter/ field leaching studies ‡	No data submitted

PEC (soil)

Parent Method of calculation	No valid DT_{50} could be determined due the nature of the active ingredient.
Application data	Crop: bean Depth of soil layer: 5 cm Soil bulk density: 1.5 g/ml % plant interception: no crop interception Number of applications: single application Application rate(s): max. 2500 g a.s./ha

Summary of initial PEC_s

Formulation/ compound	Crop	Number of applications	Maximum use rate [g product/ha]	Crop interception [%]	Effective soil exposure rate [g/ha]	PEC_S [mg/kg]
ALTHIA (Goemar)	Bean	1	2000	0	2000	2.667
AGROCEAN BASE (Agrimer)	Bean	1	2500	0	2500	3.333
STIMPLEX (Acadian)	Bean	1	2000	0	2000	2.667
KELPGROW (Asfaleia)	Bean	1	2000	0	2000	2.667
ALGAEGREEN (OGT)	Bean	1	2000	0	2000	2.667

Route and rate of degradation in water (Annex IIA, point 7.2.1)

Hydrolytic degradation of the active substance and metabolites > 10 % ‡	No data submitted
	No data submitted
	No data submitted
Photolytic degradation of active substance and metabolites above 10 % ‡	No data submitted
Quantum yield of direct phototransformation in water at Σ > 290 nm	No data submitted
Readily biodegradable ‡ (yes/no)	No data submitted

Degradation in water / sediment

Parent	No data submitted

PEC (surface water) (Annex IIIA, point 9.2.3)

Parameters used	The PECsw for all products were calculated using the Rautman spray-drift equation*, assuming a water depth of 0.3 m, vegetables (height > 50 cm) as crop.

Formulation/ compound	Crop	Number of applications	Maximum use rate [g product/ha]	PEC_{sw} [µg/L]			
				3m	5m	10m	15m
ALTHIA (Goemar)	Bean	1	2000	53.467	24.133	8.200	4.333
AGROCEAN BASE (Agrimer)	Bean	1	2500	66.833	30.167	10.250	5.417
STIMPLEX (Acadian)	Bean	1	2000	53.467	24.133	8.200	4.333
KELPGROW (Asfaleia)	Bean	1	2000	53.467	24.133	8.200	4.333
ALGAEGREEN (OGT)	Bean	1	2000	53.467	24.133	8.200	4.333

* the version of the Rautman spray-drift equation used in PECsw calculations is not available; however, results can be considered conservative and are acceptable.

Residues requiring further assessment

Environmental occurring residues requiring further assessment by other disciplines (toxicology and ecotoxicology) and or requiring consideration for groundwater exposure.	Soil: sea-algae extract Surface water: sea-algae extract Sediment: sea-algae extract Ground water: sea-algae extract Air: sea-algae extract

Monitoring data, if available (Annex IIA, point 7.4)

Soil (indicate location and type of study)	No data available
Surface water (indicate location and type of study)	No data available
Ground water (indicate location and type of study)	No data available
Air (indicate location and type of study)	No data available

Points pertinent to the classification and proposed labelling with regard to fate and behaviour data

Candidate for R 53 in the absence of data on ready biodegradability.

Ecotoxicology

Effects on terrestrial vertebrates (Annex IIA, point 8.1, Annex IIIA, points 10.1 and 10.3)

Species	Test substance	Time scale	End point (mg/kg bw)	End point (mg/kg feed)
Birds ‡				
No data available				
Mammals ‡				
Rat	Althia	Acute	LD_{50} >2000 mg/kg bw (oral and dermal)	
	Agrocean Base	Acute	LD_{50} >2000 mg/kg bw (oral and dermal)	
	Stimplex (pH 4)	Acute	LD_{50} >2000 mg/kg bw (oral and dermal)	
	Stimplex (pH 8)	Acute	LD_{50} >2000 mg/kg bw (oral and dermal)	
	Kelpgrow	Acute	LD_{50} >2000 mg/kg bw (oral and dermal)	
	Algaegreen	Acute	LD_{50} >2000 mg/kg bw (oral and dermal)	
Additional higher tier studies ‡				
No data available – not required				

Toxicity/exposure ratios for terrestrial vertebrates (Annex IIIA, points 10.1 and 10.3)

Crop and application rate: Bean (BBCH 5)
Kelpgrow, Stimplex Althia, Algaegreen: 2 kg formulated product/ha, Agrocean base (at any crop stage): 2.5 kg formulated product/ha

Indicator species/Category	Time scale	ETE	TER	Annex VI Trigger
Tier 1 (Birds)				
No data available				
Higher tier refinement (Birds)				
No data available				
Tier 1 (Mammals)				
Small omnivorous mammal[1)]	Acute	5.7 [2)]	> 17.5	10
	Long-term	Not relevant		5

Indicator species/Category	Time scale	ETE	TER	Annex VI Trigger
Higher tier refinement (Mammals)				
	Acute	Not relevant		10
	Long-term	Not relevant		5

[1)] for crops treated at BBCH < 10 the generic focal species is a small omnivorous mammal with a shortcut 90th percentile RUD = 14.3 (Table I.2, (Annex I of guidance document EFSA, 2009)

[2)] the figure refers to DDD; $DDD_{single\ application)}$= application rate x shortcut value = 0.4 kg dw a.s./ha x 14.3 = 5.7.

Worst-case acute mammal toxicity (expressed as dry matter content of the formulation) is LD_{50} > 100 mg dw a.s./kg bw/day (Althia).

Toxicity data for aquatic species (most sensitive species of each group) (Annex IIA, point 8.2, Annex IIIA, point 10.2)

Group	Test substance	Time-scale	Endpoint	Toxicity (mg preparation /L $_{nom}$)
Laboratory tests				
Fish				
Oncorhynchus mykiss	Althia	96 h	96 h NOEC (mortality)	> 100 mg/L
Danio rerio	Agrocean Base	96 h	96 h NOEC (mortality)	> 100 mg/L
Danio rerio	Stimplex (pH 4)	96 h	96 h NOEC (mortality)	> 100 mg/L
Danio rerio	Stimplex (pH 8)	96 h	96 h NOEC (mortality)	> 100 mg/L
Danio rerio	Kelpgrow	96 h	96 h NOEC (mortality)	> 100 mg/L
Aquatic invertebrates				
Daphnia magna	Althia	48 h	48 h NOEC (immobility)	> 100 mg/L
Daphnia magna	Agrocean Base	48 h	48 h NOEC (immobility)	> 100 mg/L
Daphnia magna	Stimplex (pH 4)	48 h	48 h NOEC (immobility)	> 100 mg/L
Daphnia magna	Stimplex (pH 8)	48 h	48 h NOEC (immobility)	> 100 mg/L
Daphnia magna	Kelpgrow	48 h	48 h NOEC (immobility)	> 100 mg/L
Sediment dwelling organisms				
No data available – not required				
Algae				
Scenedesmus subspicatus	Althia	72 h	72 h EC10 (biomass and growth rate)	> 100 mg/L
Pseudokirchneriella subcapitata	Agrocean Base	72 h	72 h EC10 (biomass and growth rate)	> 100 mg/L
Pseudokirchneriella subcapitata	Stimplex (pH 4)	72 h	72 h EC10 (biomass and growth rate)	> 30 mg/L
Pseudokirchneriella subcapitata	Stimplex (pH 8)	72 h	72 h EC10 (biomass and growth rate)	> 30 mg/L
Pseudokirchneriella subcapitata	Kelpgrow	72 h	72 h EC10 (biomass and growth rate)	> 100 mg/L

Pseudokirchneriella subcapitata	Algaegreen	72 h	72 h EC10 (biomass and growth rate)	> 30 mg/L
Higher plants				
No data available –Data gap				
Microcosm or mesocosm tests				
No data available – not required				

Toxicity/exposure ratios for the most sensitive aquatic organisms (Annex IIIA, point 10.2)

Crop and application rate: Bean (BBCH 5) Kelpgrow, Stimplex, Althia, Alagegreen: 2 kg formulated product/ha, Agrocean base (at any crop stage): 2.5 kg formulated product/ha

Test substance	Organism	Toxicity end point (mg/L)	Time scale	PEC_i*	TER	Annex VI Trigger
Althia	Fish		Acute	53.47	> 1870	100
Althia	Aquatic invertebrates		Acute	53.47	> 1870	100
Althia	Algae		Chronic	53.47	> 1870	10
Agrocean Base	Fish		Acute	66.83	> 1496	100
Agrocean Base	Aquatic invertebrates		Acute	66.83	> 1496	100
Agrocean Base	Algae		Chronic	66.83	> 1496	10
Stimplex	Fish		Acute	53.47	> 1870	100
Stimplex	Aquatic invertebrates		Acute	53.47	> 1870	100
Stimplex	Algae		Chronic	53.47	> 561	10
Kelpgrow	Fish		Acute	53.47	> 1870	100
Kelpgrow	Aquatic invertebrates		Acute	53.47	> 1870	100
Kelpgrow	Algae		Chronic	53.47	> 1870	10
Algaegreen	Algae		Chronic	53.47	> 561	10
a.s.	Higher plants		Chronic	Data gap	-	10
a.s.	Sediment-dwelling organisms		Chronic	Not available	Not relevant	10

* Global maximum PEC (µg/L) due to the spray drift (3m).

Bioconcentration				
	Active substance	Metabolite1	Metabolite2	Metabolite3
$logP_{O/W}$	No data available			

Bioconcentration				
Bioconcentration factor (BCF)	No data available - not required			
Annex VI Trigger for the bioconcentration factor				
Clearance time (days) (CT_{50})				
(CT_{90})				
Level and nature of residues (%) in organisms after the 14 day depuration phase				

Effects on honeybees (Annex IIA, point 8.3.1, Annex IIIA, point 10.4)

Test substance	Acute oral toxicity (LD_{50} µg/bee)	Acute contact toxicity (LD_{50} µg/bee)
a.s.	No data available	No data available
formulation	No data available	No data available
Field or semi-field tests		
No data available		

Hazard quotients for honey bees (Annex IIIA, point 10.4)

Test substance	Route	Hazard quotient	Annex VI Trigger
a.s.	Contact	Not relevant	50
a.s.	oral	Not relevant	50
formulation	Contact	Not relevant*	50
formulation	oral	Not relevant*	50

*: data gap for the use with the formulation Agrocean base for the case(s) when bees can be exposed.

Effects on other arthropod species (Annex IIA, point 8.3.2, Annex IIIA, point 10.5)

Laboratory tests with standard sensitive species

Species	Test Substance	End point	Effect (LR_{50} g/ha)
Typhlodromus pyri ‡		Mortality	No data available
Aphidius rhopalosiphi ‡		Mortality	No data available

Test substance	Species	Effect (LR_{50} g/ha)	HQ in-field	HQ off-field	Trigger
	Typhlodromus pyri	No data available	data gap	data gap	2

Test substance	Species	Effect (LR_{50} g/ha)	HQ in-field	HQ off-field	Trigger
	Aphidius rhopalosiphi	No data available	data gap	data gap	2

Further laboratory and extended laboratory studies ‡

Species	Life stage	Test substance, substrate and duration	Dose (g/ha)	End point	% effect	Trigger value
				No data available		50 %

Field or semi-field tests: no reliable data are available

Effects on earthworms, other soil macro-organisms and soil micro-organisms (Annex IIA points 8.4 and 8.5. Annex IIIA, points, 10.6 and 10.7)

Test organism	Test substance	Time scale	End point
Earthworms			
No data available			
Other soil macro-organisms			
No data available			
Collembola			
No data available			
Soil micro-organisms			
No data available			
Field studies			
No data available			

Data gap is identified to address the risk for soil organisms (earthworms, soil macro- and micro- organisms).

Toxicity/exposure ratios for soil organisms

Test organism	Test substance	Time scale	Soil PEC	TER	Trigger
Earthworms					
Eisenia foetida	a.s. ‡			data gap	10
Other soil macro-organisms					
Soil mite	a.s. ‡			data gap*	
	formulation				
	Metabolite 1				
Collembola	a.s. ‡			data gap*	
	formulation				

Test organism	Test substance	Time scale	Soil PEC	TER	Trigger
	Metabolite 1				

*: the relevant data gap is a general data gap to address the risk for soil organisms

Effects on non target plants (Annex IIA, point 8.6, Annex IIIA, point 10.8)

Preliminary screening data

No data available

Laboratory dose response tests

Most sensitive species	Test substance	ER_{50} $(g/ha)^2$ vegetative vigour	ER_{50} (g/ha) emergence	Exposure (g/ha)	TER	Trigger
No data available - data gap						

Additional studies (e.g. semi-field or field studies)

No data available

Effects on biological methods for sewage treatment (Annex IIA 8.7)

Test type/organism	End point
Activated sludge	No data available

Ecotoxicologically relevant compounds (consider parent and all relevant metabolites requiring further assessment from the fate section)

Compartment	
soil	sea-algae extracts
water	sea-algae extracts
sediment	sea-algae extracts
groundwater	sea-algae extracts

Classification and proposed labelling with regard to ecotoxicological data (Annex IIA, point 10 and Annex IIIA, point 12.3)

	RMS/peer review proposal
Active substance	No classification is proposed

APPENDIX B - DETAILED SPECIFICATION OF THE MARKER COMPOUNDS IN THE FORMULATIONS

ACADIAN

Sea-algae Extract of *Ascophyllum nodosum* (STIMPLEX)

Table 1 Summary of the specification for STIMPLEX based on wet weight analysis

	Proposed Specification g/kg
Mannitol	6.0
Fucoidans	19.0
Alginic acids	24.0
Water and unidentified components	Up to 1000

Table 2 Summary of the specification for STIMPLEX based on dry weight analysis

	Proposed Specification g/kg
Mannitol	30.0
Fucoidans	99.0
Alginic acids	124.0

AGRIMER

Sea-algae Extract of *Laminaria digitata* (AGROCEAN BASE)

Table 3 Summary of the specification for AGROCEAN BASE based on wet weight analysis

	Proposed Specification g/kg
Mannitol	18.0
Fucoidans	3.5
Alginic acids	18.0
Water and unidentified components	Up to 1000

Table 4 Summary of the specification for AGROCEAN BASE based on dry weight analysis

	Proposed Specification g/kg
Mannitol	166.0
Fucoidans	34.0
Alginic acids	166.5

ASFALEIA

Sea-algae Extract of *Macrocystis integrifolia* (KELPGROW)

Table 5 Summary of the specification for KELPGROW based on wet weight analysis

	Proposed Specification g/kg
Mannitol	6.0
Fucoidans	2.0
Alginic acids	1.5
Water and unidentified components	Up to 1000

Table 6 Summary of the specification for KELPGROW based on dry weight analysis

	Proposed Specification g/kg
Mannitol	191.0
Fucoidans	67.0
Alginic acids	23.5

GOËMAR

Sea-algae Extract of *Laminaria digitata* and *Ascophyllum nodosum* (ALTHIA)

Table 7 Summary of the specification for ALTHIA based on wet weight analysis.

	Proposed Specification g/kg
Mannitol	7.3
Fucoidans	2.8
Alginic acids	1
Water and unidentified components	Up to 1000

Table 8 Summary of the specification for ALTHIA based on dry weight analysis.

	Proposed Specification g/kg
Mannitol	147
Fucoidans	55.5
Alginic acids	18.5

OILEAN GLAS

SEA ALGAE EXTRACT of Ascophyllum sp. (ALGAEGREEN)

Table 9 Summary of the specification for ALGAEGREEN base on wet weight analysis

	Proposed Specification g/kg
Mannitol	6.0
Fucoidans	3.0
Alginic acids	6.0
Water and unidenfied components	Up to 1000

Table 10 Summary of the specification for ALGAEGREEN base on dry weight analysis

	Proposed Specification g/kg
Mannitol	140.0
Fucoidans	38.0
Alginic acids	77.0

ABBREVIATIONS

1/n	slope of Freundlich isotherm
λ	wavelength
ε	decadic molar extinction coefficient
°C	degree Celsius (centigrade)
µg	microgram
µm	micrometer (micron)
a.s.	active substance
AChE	acetylcholinesterase
ADE	actual dermal exposure
ADI	acceptable daily intake
AF	assessment factor
AOEL	acceptable operator exposure level
AP	alkaline phosphatase
AR	applied radioactivity
ARfD	acute reference dose
AST	aspartate aminotransferase (SGOT)
AV	avoidance factor
BCF	bioconcentration factor
BUN	blood urea nitrogen
bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming units
ChE	cholinesterase
CI	confidence interval
CIPAC	Collaborative International Pesticides Analytical Council Limited
CL	confidence limits
cm	centimetre
d	day
DAA	days after application
DAR	draft assessment report
DAT	days after treatment
DM	dry matter
DT_{50}	period required for 50 percent disappearance (define method of estimation)
DT_{90}	period required for 90 percent disappearance (define method of estimation)
dw	dry weight
EbC_{50}	effective concentration (biomass)
EC_{50}	effective concentration
ECHA	European Chemical Agency
EEC	European Economic Community
EINECS	European Inventory of Existing Commercial Chemical Substances
ELINCS	European List of New Chemical Substances
EMDI	estimated maximum daily intake
ER_{50}	emergence rate/effective rate, median
ErC_{50}	effective concentration (growth rate)
EU	European Union
EUROPOEM	European Predictive Operator Exposure Model
f(twa)	time weighted average factor
FAO	Food and Agriculture Organisation of the United Nations
FIR	Food intake rate
FOB	functional observation battery
FOCUS	Forum for the Co-ordination of Pesticide Fate Models and their Use
g	gram
GAP	good agricultural practice

GC	gas chromatography
GCPF	Global Crop Protection Federation (formerly known as GIFAP)
GGT	gamma glutamyl transferase
GM	geometric mean
GS	growth stage
GSH	glutathion
h	hour(s)
ha	hectare
Hb	haemoglobin
Hct	haematocrit
hL	hectolitre
HPLC	high pressure liquid chromatography or high performance liquid chromatography
HPLC-MS	high pressure liquid chromatography – mass spectrometry
HQ	hazard quotient
IEDI	international estimated daily intake
IESTI	international estimated short-term intake
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
JMPR	Joint Meeting on the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues (Joint Meeting on Pesticide Residues)
K_{doc}	organic carbon linear adsorption coefficient
kg	kilogram
K_{Foc}	Freundlich organic carbon adsorption coefficient
L	litre
LC	liquid chromatography
LC_{50}	lethal concentration, median
LC-MS	liquid chromatography-mass spectrometry
LC-MS-MS	liquid chromatography with tandem mass spectrometry
LD_{50}	lethal dose, median; dosis letalis media
LDH	lactate dehydrogenase
LOAEL	lowest observable adverse effect level
LOD	limit of detection
LOQ	limit of quantification (determination)
m	metre
M/L	mixing and loading
MAF	multiple application factor
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
mg	milligram
mL	millilitre
mm	millimetre
mN	milli-newton
MRL	maximum residue limit or level
MS	mass spectrometry
MSDS	material safety data sheet
MTD	maximum tolerated dose
MWHC	maximum water holding capacity
NESTI	national estimated short-term intake
ng	nanogram
NOAEC	no observed adverse effect concentration
NOAEL	no observed adverse effect level
NOEC	no observed effect concentration

NOEL	no observed effect level
OM	organic matter content
Pa	pascal
PD	proportion of different food types
PEC	predicted environmental concentration
PEC_{air}	predicted environmental concentration in air
PEC_{gw}	predicted environmental concentration in ground water
PEC_{sed}	predicted environmental concentration in sediment
PEC_{soil}	predicted environmental concentration in soil
PEC_{sw}	predicted environmental concentration in surface water
pH	pH-value
PHED	pesticide handler's exposure data
PHI	pre-harvest interval
PIE	potential inhalation exposure
pK_a	negative logarithm (to the base 10) of the dissociation constant
P_{ow}	partition coefficient between *n*-octanol and water
PPE	personal protective equipment
ppm	parts per million (10^{-6})
ppp	plant protection product
PT	proportion of diet obtained in the treated area
PTT	partial thromboplastin time
QSAR	quantitative structure-activity relationship
r^2	coefficient of determination
RPE	respiratory protective equipment
RUD	residue per unit dose
SC	suspension concentrate
SD	standard deviation
SFO	single first-order
SL	soluble concentrate
SSD	species sensitivity distribution
STMR	supervised trials median residue
$t_{1/2}$	half-life (define method of estimation)
TER	toxicity exposure ratio
TER_A	toxicity exposure ratio for acute exposure
TER_{LT}	toxicity exposure ratio following chronic exposure
TER_{ST}	toxicity exposure ratio following repeated exposure
TK	technical concentrate
TLV	threshold limit value
TMDI	theoretical maximum daily intake
TRR	total radioactive residue
TSH	thyroid stimulating hormone (thyrotropin)
TWA	time weighted average
UDS	unscheduled DNA synthesis
UV	ultraviolet
W/S	water/sediment
w/v	weight per volume
w/w	weight per weight
WBC	white blood cell
WHO	World Health Organisation
wk	week
yr	year

BioAtlantis

Nature Working Naturally™

MATERIAL SAFETY DATA SHEET

BioAtlantis AgriPrime Nematec®

1. Company & Product Identification

Company Identifier:

Manufacturer: BioAtlantis Ltd.
Address: Kerry Technology Park, Tralee, Co. Kerry, Ireland.
Contact Details: Tel: +353 (0) 667 118 477, Fax: +353 (0) 667 119 802, E-mail: info@bioatlantis.com
Emergency Phone No.: Tel: +353 (0) 667 118 477
Emergency Contact Name: TJ Greaney
Emergency Contact Times: Monday - Friday, 9am – 5pm (GMT)

Product Identifier:

Product Name: AgriPrime Nematec®
Product Code: A037
Description: *Laminaria* extract.
EC number: 289-980-0
CAS number: 90046-12-1
Intended Use: Plant strengthener/organic fertilizer/ Foliar and Fertigation applications in agriculture and horticulture.

2. Hazard Identification

Classification: Non-hazardous
Signal word: No signal word
Precautionary statements: If skin irritation occurs: Get medical advice/attention
Avoid breathing spray
Use personal protective equipment as required
Keep out of the reach of children
Other hazards: This product will stain skin upon prolonged exposure

3. Composition / Information on Ingredients

Substance Name:	Concentration	Classification regulation (EC) No 1272/2008	Classification (Directive 1999/45/EC)	CAS number:	EC number:
Laminaria, extract	50%	Non-hazardous	Non-hazardous	90046-12-1	289-980-0

Note: Confidential business information has been omitted.

4. First-Aid Measures

General: Non-hazardous material. If irritation occurs, seek medical advice.

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Email: info@bioatlantis.com. Web: www.bioatlantis.com

Inhalation: Remove the exposed individual from the area into fresh air is recommended.
Skin: Remove contaminated clothing. Wash thoroughly with soap and water. Seek medical attention if irritation occurs.
Eyes: In case of contact, irrigate with clean water for >15 minutes. Seek medical attention if irritation or redness persists.
Ingestion: If swallowed drink plenty of water or milk. Seek medical advice. Do not induce vomiting.
First Aid Responders: Wear rubber or nitrile gloves to prevent staining of skin.

5. Fire-Fighting Measures

General: Non-flammable and non-explosive.
Protective action: In the event of a fire, keep containers cool with water spray.
Unusual fire/explosion hazards: Unknown.
Special protective equipment: Fire fighters should wear personal protective equipment to prevent contamination of skin, eyes and personal clothing.
Caution: Slippery in liquid form or when mixed with water.

6. Accidental Release Measures

Safety Precautions: Use personal protective equipment to prevent contamination of skin, eyes and clothing.
Small spills: May be washed to drain.
Large spills: Contain spill with sand, sawdust, absorbent clay or similar material. Sweep up and transfer material into labelled containers for transfer to a safe place for disposal. Flush the area with plenty of water when all the spillage has been removed. This product will stain concrete.
Drainage: Prevent the spillage from entering watercourses, sewers or drains if possible. Inform the authorities if gross contamination of water is threatened or occurs.

7. Handling and Storage

Precautions for safe handling:

- Eating, drinking and smoking in work areas is prohibited.
- Use personal protective equipment to prevent contamination of skin, eyes and personal clothing.
- Avoid breathing mist/spray.
- If irritation occurs: Get medical advice/attention.
- Keep out of reach of children.
- Avoid excessive handling which may cause staining of skin.
- Wash your hands after use.

Precautions for safe storage:

- Keep in original container, tightly closed.
- Store in a suitable cool, dry location, out of direct sunlight, away from direct sources of heat.
- Protect from frost.
- Keep away from food, drink and animal feeding stuffs.

8. Exposure Controls/Personal Protection

Engineering Controls: Advisable to spray product in a well-ventilated area. Do not intentionally breathe dust/fume/gas/mist/vapours/spray.
Respiratory Protection: No respirator is required.

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Hand Protection: Wear rubber or nitrile gloves to prevent staining of skin.
Skin Protection: Wear a water impermeable bodysuit to prevent staining of skin.
Eye Protection: Wear chemical goggles. Eyewash facilities should be available.

9. Physical and Chemical Properties

Appearance:	Brown-greenish liquid
Odour:	Sweetish, marine
Odour Threshold:	Not determined
pH:	3.5 – 5.5
Melting Point/Freezing Point:	Not determined
Initial Boiling Point/Boiling Range:	Data lacking
Flash Point:	N/A
Evaporation Rate:	Not determined
Flammability:	Non flammable
Upper/lower flammability or exposure limits:	Not determined
Vapour Pressure:	Not determined
Vapour Density:	Not determined
Relative Density:	1.21 – 1.25
Solubility:	≥98.5% water soluble
Partition Coefficient: n-octanol/water:	Not determined
Auto-ignition Temperature:	550^{o}C
Decomposition Temperature:	Not determined
Viscosity:	<300 cP

10. Stability and Reactivity

General: Stable under recommend storage conditions. It can be stored for 2 years in unopened containers.
Incompatible Materials: Do not mix with highly acidic chemicals. Oxidants and acids may cause degradation.
Conditions to Avoid: Avoid direct sunlight, protect against excessive heat and freezing.
Hazardous Decomposition Products: N/A

11. Toxicological Information

Acute oral toxicity:	Ingestion of large amounts may induce nausea.
Skin corrosion/irritation:	No sensitisation effects have been observed
Serious eye damage/irritation:	Data lacking
Respiratory of skin sensitisation:	Data lacking
Germ cell mutagenicity:	Data lacking
Carcinogenicity:	Data lacking
Reproductive toxicity:	Data lacking
STOT – single exposure:	Data lacking
STOT – repeated exposure:	Data lacking
Aspiration hazard:	No sensitisation effects have been observed

12. Ecological Information

Ecotoxicity: High concentrations may cause scorching in plants. Not toxic to vertebrates, invertebrates or marine algae.
Persistence and Degradability: Components are readily biodegradable.
Bioaccumulative Potential: Biodegradable. Product presents minimal environmental impact.

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Spillages: Prevent the spillage from entering watercourses, sewers or drains if possible. Inform the authorities if gross contamination of water is threatened or occurs.

13. Disposal Considerations

Product Disposal: Consult the local authorities for advice on disposal of unwanted or waste product.
Packaging Disposal: Dispose of containers as local regulations allow.
Personal Protection: Person carrying out disposal should wear PVC gloves.
Caution: Do not contaminate surface waters or ditches with product or used containers.

14. Transport Information

General: Non-hazardous material. No special precautions required.
Road Transport: Not hazardous
UN Number: No UN Number
Proper Shipping Name: BioAtlantis Liquid Seaweed Extract Concentrate.
IMDG Class: Not applicable
CPL Packing Group: Not applicable

15. Regulatory Information

Composition: *Laminaria* extract
H-Statements: None
P-Statements: P102: Keep out of reach of children
P261: Avoid breathing spray
P281: Use personal protective equipment as required
P332/313: If skin irritation occurs: Get medical advice/attention
R-Phrases: None
S-Phrases: S2: Keep out of the reach of children
S23: Do not breathe spray
S36/37/39: Wear suitable protective clothing, gloves and eye/face protection

16. Other information

The above information is intended to give health and safety guidance on the storage and transport of the substance or product to which it relates. It is not intended to apply to the use of the product, for which purpose the product label and any appropriate technical usage literature available should be consulted and any relevant licences, consents or approvals complied with. The requirements or recommendations of any relevant site or working procedure, system or policy in force or arising from any risk assessment involving the substance or product should take precedence over any of the guidance contained in this safety data sheet where there is a difference in the information given. The information provided in this safety data sheet is accurate to the best of the issuer's available knowledge at the date of publication, and will be updated as and when appropriate. No liability will be accepted for any loss or damage resulting from any failure to take account of information or advice contained in this safety data sheet.

End of Material Safety Data Sheet

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BioAtlantis

Nature Working Naturally™

MATERIAL SAFETY DATA SHEET

BioAtlantis Ecolicitor®

1. Company & Product Identification

Company Identifier:

Manufacturer:	BioAtlantis Ltd.
Address:	Kerry Technology Park, Tralee, Co. Kerry, Ireland.
Contact Details:	Tel: +353 (0) 667 118 477, Fax: +353 (0) 667 119 802, E-mail: info@bioatlantis.com
Emergency Phone No.:	Tel: +353 (0) 667 118 477
Emergency Contact Name:	Bryan Corridan
Emergency Contact Times:	Monday - Friday, 9am – 5pm (GMT)

Product Identifier:

Product Name:	Ecolicitor®
Product Code:	A012
Synonyms:	Ocean Green Ecolicitor®, Ascogold, Algagold
Description:	*Ascophyllum nodosum,* extract.
EC number:	283-907-6
CAS number:	84775-78-0
Intended Use:	Plant growth biostimulant/organic fertilizer/foliar spray for use in agriculture and horticulture.

2. Hazard Identification

Classification:	Non-hazardous
Signal word:	No signal word
Precautionary statements:	If skin irritation occurs: Get medical advice/attention Avoid breathing spray Use personal protective equipment as required Keep out of the reach of children
Other hazards:	This product will stain skin upon prolonged exposure

3. Composition / Information on Ingredients

Substance Name:	*Ascophyllum nodosum,* extract
Substance Description:	30% w/v soluble seaweed extract from *Ascophyllum nodosum* brown algae
Note:	Confidential business information has been omitted
EC number:	283-907-6
CAS number:	84775-78-0

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Email: info@bioatlantis.com. Web: www.bioatlantis.com

4. First-Aid Measures

General:	Non-hazardous material. If irritation occurs, seek medical advice.
Inhalation:	Remove the exposed individual from the area to fresh air is recommended.
Skin:	Remove contaminated clothing. Wash thoroughly with soap and water. Seek medical attention if irritation occurs.
Eyes:	In case of contact, irrigate with clean water for >15 minutes. Seek medical attention if irritation or redness persists.
Ingestion:	If swallowed drink plenty of water or milk. Seek medical advice. Do not induce vomiting.
First Aid Responders:	Wear rubber or nitrile gloves to prevent staining of skin.

5. Fire-Fighting Measures

General:	Non-flammable and non-explosive.
Protective action:	In the event of a fire, keep containers cool with water spray.
Unusual fire/explosion hazards:	Unknown.
Special protective equipment:	Fire fighters should wear personal protective equipment to prevent contamination of skin, eyes and personal clothing.
Caution:	Slippery in liquid form or when mixed with water

6. Accidental Release Measures

Safety Precautions:	Use personal protective equipment to prevent contamination of skin, eyes and clothing.
Small spills:	May be washed to drain.
Large spills:	Contain spill with sand, sawdust, absorbent clay or similar material. Sweep up and transfer material into labelled containers for transfer to a safe place for disposal. Flush the area with plenty of water when all the spillage has been removed. This product will stain concrete.
Drainage:	Prevent the spillage from entering watercourses, sewers or drains if possible. Inform the authorities if gross contamination of water is threatened or occurs.

7. Handling and Storage

Precautions for safe handling:

- Eating, drinking and smoking in work areas is prohibited.
- Use personal protective equipment to prevent contamination of skin, eyes and personal clothing.
- Avoid breathing mist/spray.
- If irritation occurs: Get medical advice/attention.
- Keep out of reach of children.
- Avoid excessive handling which may cause staining of skin.
- Wash your hands after use.

Precautions for safe storage:

- Keep in original container, tightly closed.
- Store in a suitable cool, dry location, out of direct sunlight, away from direct sources of heat.
- Protect from frost.
- Keep away from food, drink and animal feeding stuffs.

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8. Exposure Controls/Personal Protection

Engineering Controls: Advisable to spray product in a well-ventilated area. Do not intentionally breathe dust/fume/gas/mist/vapours/spray.
Respiratory Protection: No respirator is required.
Hand Protection: Wear rubber or nitrile gloves to prevent staining of skin.
Skin Protection: Wear a water impermeable bodysuit to prevent staining of skin.
Eye Protection: Wear chemical goggles. Eyewash facilities should be available.

9. Physical and Chemical Properties

Appearance:	Black/brown liquid
Odour:	Marine
Odour Threshold:	Not determined
pH:	3.8 – 4.8
Melting Point/Freezing Point:	Not determined
Initial Boiling Point/Boiling Range:	Data lacking
Flash Point:	N/A
Evaporation Rate:	Not determined
Flammability:	Non flammable
Upper/lower flammability or exposure limits:	Not determined
Vapour Pressure:	Not determined
Vapour Density:	Not determined
Relative Density:	1.13 – 1.17 Kg/L
Solubility:	≥95% soluble
Partition Coefficient: n-octanol/water:	Not determined
Auto-ignition Temperature:	550^{o}C
Decomposition Temperature:	Not determined
Viscosity:	<300 cP

10. Stability and Reactivity

General: Stable under recommend storage conditions. It can be stored for 2 years in unopened containers.
Incompatible Materials: Do not mix with highly acidic chemicals. Oxidants and acids may cause degradation.
Conditions to Avoid: Avoid direct sunlight, protect against excessive heat and freezing.
Hazardous Decomposition Products: N/A

11. Toxicological Information

Acute oral toxicity:	Ingestion of large amounts may induce nausea.
Skin corrosion/irritation:	No sensitisation effects have been observed.
Serious eye damage/irritation:	Data lacking
Respiratory of skin sensitisation:	Data lacking
Germ cell mutagenicity:	Data lacking
Carcinogenicity:	Data lacking
Reproductive toxicity:	Data lacking
STOT – single exposure:	Data lacking
STOT – repeated exposure:	Data lacking
Aspiration hazard:	No sensitisation effects have been observed.

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12. Ecological Information

Ecotoxicity: High concentrations may cause scorching in plants. Not toxic to vertebrates, invertebrates or marine algae.
Persistence and Degradability: Components are readily biodegradable.
Bioaccumulative Potential: Biodegradable. Product presents minimal environmental impact.
Spillages: Prevent the spillage from entering watercourses, sewers or drains if possible. Inform the authorities if gross contamination of water is threatened or occurs.

13. Disposal Considerations

Product Disposal: Consult the local authorities for advice on disposal of unwanted or waste product.
Packaging Disposal: Dispose of containers as local regulations allow.
Personal Protection: Person carrying out disposal should wear PVC gloves.
Caution: Do not contaminate surface waters or ditches with product or used containers.

14. Transport Information

General: Non-hazardous material. No special precautions required.
Road Transport: Not hazardous
UN Number: No UN Number
Proper Shipping Name: BioAtlantis Liquid Seaweed Extract Concentrate.
IMDG Class: Not applicable
CPL Packing Group: Not applicable

15. Regulatory Information

Composition: *Ascophyllum nodosum,* extract
H-Statements: None
P-Statements: P102: Keep out of reach of children
P261: Avoid breathing spray
P281: Use personal protective equipment as required
P332/313: If skin irritation occurs: Get medical advice/attention
R-Phrases: None
S-Phrases: S2: Keep out of the reach of children
S23: Do not breathe spray
S36/37/39: Wear suitable protective clothing, gloves and eye/face protection

16. Other information

The above information is intended to give health and safety guidance on the storage and transport of the substance or product to which it relates. It is not intended to apply to the use of the product, for which purpose the product label and any appropriate technical usage literature available should be consulted and any relevant licences, consents or approvals complied with. The requirements or recommendations of any relevant site or working procedure, system or policy in force or arising from any risk assessment involving the substance or product should take precedence over any of the guidance contained in this safety data sheet where there is a difference in the information given. The information provided in this safety data sheet is accurate to the best of the issuer's available knowledge at the date of publication, and will be updated as and when appropriate. No liability will be accepted for any loss or damage resulting from any failure to take account of information or advice contained in this safety data sheet.

End of Material Safety Data Sheet

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