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# Chemical signaling and defense in brown algal kelps during interactions with herbivores

Léa Cabioch

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*Equipe Biologie des algues et interactions avec l'environnement*

**Chemical signaling and defense in brown algal kelps  
during interactions with herbivores**

Par Léa Cabioch

Thèse de doctorat en Biologie Marine

Dirigée par Catherine Leblanc et Sylvain Faugeron

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Devant un jury composé de :

Dr. Bernard Banaigs (CRIOBE, Perpignan)	Rapporteur
Dr. Florian Weinberger (Geomar, Kiel)	Rapporteur
Pr. Sylvie Baudino (LBVPAM, St Etienne)	Examinatrice
Pr. Evie Wieters (PUC, Santiago de Chile)	Représentante PUC
Pr. Christophe Destombe (CNRS-UPMC, Roscoff)	Représentant UPMC
Dr. Catherine Leblanc (CNRS-UPMC, Roscoff)	Directrice de thèse
Dr. Sylvain Faugeron (PUC, Santiago de Chile)	Co-directeur de thèse





PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE



UNIVERSITÉ PIERRE ET MARIE CURIE (PARIS VI)

## TESIS DE DOCTORADO

### **Señalización química y defensa en las algas pardas en interacción con los herbívoros**

Para optar al grado académico de  
Docteur de l'Université Pierre & Marie Curie  
y  
al grado de Doctor en Ciencias Biológicas Mención Ecología  
de la Pontificia Universidad Católica de Chile

Presentada por

**Léa Cabioch**

Directores de tesis: Catherine Leblanc, Universidad Pierre et Marie Curie  
Sylvain Faugeron, Pontificia Universidad Católica de Chile

Comisión de tesis: Dr. Bernard Banaigs (CRIOBE, Perpignan)  
Dr. Florian Weinberger (Geomar, Kiel)  
Pr. Sylvie Baudino (LBVPAM, St Etienne)  
Pr. Evie Wieters (PUC, Santiago de Chile)  
Pr. Christophe Destombe (CNRS-UPMC, Roscoff)

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*A mes parents*

*Qui ont éveillé mon goût pour la science,*

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*Et qui auront réussi le défi de m'amener jusqu'au bout d'une thèse.*



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# **General introduction**

# I. Chemical communication in plant/herbivore interactions

## 1. Basic concepts of Chemical Ecology

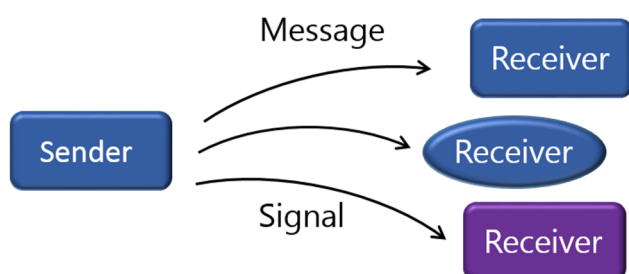
### a. Chemical ecology: definition and main questions

Odors are integral part of our everyday lives. With the senses of view and hearing, olfaction gives lots of information on our surroundings, whether for detection of food sources, our close relatives or even for finding mates and reproducing. To fulfill these vital functions, each living being constantly interact with its abiotic environment like temperature, humidity or nutrient availability, and with its biotic environment by perception of the presence of neighboring living organisms. According to its environment, an organism forms a chemosphere around it, composed of variable volatile blends. As constituted by a wide range of chemical compounds with different relative concentrations, each organism possesses its own scent. The chemical compounds composing the chemosphere of an individual can then be perceived by surrounding living beings, thus influencing the behavior of neighboring organisms, resulting either in connections between organisms or, on the contrary, in repulsion. In the end, chemical communications between organisms shape communities, involving microorganisms like bacteria or viruses, as well as macro-organisms such as animals, plants or fungi.

Investigations of the chemical interactions between living organisms and their environment belong to the domain of Chemical Ecology. At the interface between Ecology and Chemistry, Chemical Ecology could be defined as the art of deciphering the language of the Nature. In other words, this research domain aims at understanding the distribution and abundance of organisms, their complex biotic (mutualism and parasitism, prey-predator relationships, community assembly) and abiotic interactions, through mediation by chemical compounds in different spatial and temporal scales (Amsler, 2012; Raguso et al., 2015). The main questions asked in chemical ecology are relative to either chemistry, or ecology, or even a mix between the two (Heil, 2014): what is the nature of the chemical signals involved in the communication? What are their impacts on the interaction with grazers/pollinators/parasites? What is the evolutionary origin of chemical compounds and how does their emission affect the fitness of emitters and receivers? How does it explain the community structure of the organisms? Are volatile compounds co-evolved signals or simply cues that other organisms use to determine the defensive status of a plant? Are chemical signals just the physiological consequence of internal metabolic resistance-related processes? Does the emitter benefit from warning its neighbors? How do plants release and perceive chemical compounds?

b. The chemical message: emission and perception

Basically, communication is defined as an emission of a message by a sender, which can be perceived by and can modify the behavior of one or more receiver(s) (Fig 1). A distinction was proposed to differentiate two types of information: a simple emitted information, named message, and the perceived and interpreted information named signal (Lindström and Kotiaho, 2002; Soler, 2010). Raguso et al. (2015) added that, “by convention, signals are distinguished from cues in that they confer fitness benefits to the source organism (sender) by altering the behavior of the receiver, and are inferred to have evolved in the context of the sender-receiver interaction”. Signals can be used in intraspecific (i.e. between organisms of the same species) as well as in interspecific interactions, thus allowing pollinator attraction, herbivore deterrence or warning conspecifics of a danger (Lindström and Kotiaho, 2002).



**Figure 1.** Schematic representation of message transduction between a sender and one or more receiver(s). A simple information can be distinguished from a signal which triggers a modification in the behavior of the receiver. The signal can either be involved in intraspecific or in interspecific interactions.

Living organisms can interact with each other by a wide variety of relationships, involving specific chemical dialogues between partners. For example, species living in symbiosis emit compounds which provide benefits for the other organism, while parasitism, predation or competition involve toxic, repellent or digestion-affecting chemical cues (Raguso et al., 2015). In mutualistic interactions, specific partners are involved and submitted to reciprocal selective pressures, meanwhile avoiding parasites from taking advantages of this chemical communication. This co-adaptation between the sender and the receiver finally shapes the specific interaction. Mutualism thus engages exchanges of chemical compounds specifically deciphered by these two protagonists, but can be detected by any other biological entity. It seems that chemical communications between microorganisms and between them and their environment probably evolved well before terrestrial plants colonized Earth (Mithöfer and Boland, 2016). They likely served first for sensing the environment and finding nutrients and mates. Then, these communication systems may have evolved in plants and in other complex multicellular lineages, from unicellular ancestral Eukaryotes. Thus, for all living organisms, chemical communication is an essential vector of the information, a central and crucial component for interaction between organisms for diverse species, genus or phylum. Among the diverse chemical mediators, Volatile



Organic Compounds (VOCs) are characterized by low molecular weight and low to moderate hydrophilicity (Fink 2007). VOCs produced by marine and land plants are released into the atmosphere, where they are extremely reactive, their life-times varying from minutes to hours (Maffei, 2010). In land plants, they comprise a wide range of chemicals, like terpenes (homo-, mono-, di-, sesquiterpenoids), fatty acid derived C6-volatiles, amino acids and their derivatives, phenylpropanoids such as methyl salicylate (MeSA), as well as certain alkanes, alkenes, alcohols, esters, aldehydes, and ketones (Holopainen and Gershenzon, 2010; Maffei, 2010). The composition of the blends varies according to the plant species, the organ, the developmental stage and environmental conditions (Holopainen and Gershenzon, 2010).

### c. Roles of chemical communication in plants

This is now well established that land plants can perceive the abiotic and biotic variations of their environment and respond accordingly. Chemical signals are involved in numerous processes of plant life, such as defense against herbivores and pathogens, as well as attraction of pollinators, seed dispersers and other beneficial animals and microorganisms. VOCs also serve as signals in plant–plant communication (Dudareva and Pichersky, 2008) and sometimes as wound sealers (Maffei, 2010). Chemical communication is well known in attracting pollinators in the mutualistic interaction between the fig tree and its specific pollinators. It has been recognized that one *Ficus* species emits species-specific scents that usually attract a single species of pollinator when fig flowers are ready for pollination, the receptive phase (Grison et al., 1999; Proffit and Johnson, 2009). More recently, the link between the attraction of pollinators and modifications of the bouquet of volatiles has been described in the fig tree *Ficus septica* and its specialist pollinator (Conchou et al., 2014; Appendix 1). Indeed, receptive fig flowers emit a specific ratio of chemical compounds, which attracts pollinators to flowers. After pollination, fig flowers were shown to emit a scent different from the receptivity phase, which would indicate to pollinators that flowers are no longer interesting for them. Moreover, two species of pollinators coexist on the same *Ficus* species, and it appears that, for the activity period of each pollinator species, the scent emitted by receptive figs is different. Thus, the two pollinator species could be exposed to different receptive fig odors, shaping the mutualistic interaction between the fig tree and its pollinators (Conchou et al., 2014). Similarly, it was shown that seed dispersion in fig tree was driven by a specific blend of chemical compounds, attracting bats and other dispersal agents during their activity period (Borges et al., 2011). In *Ficus benghalensis*, scents emitted by ripe fruits are different between day and night, allowing seed dispersion by bats at night and by birds during the day. Therefore, mutualistic interactions between a plant species and beneficial animals are mostly shaped by emission of infochemicals, giving indications on its physiological state.

As they are sessile, land plants have to deploy defenses against abiotic and biotic stresses. Besides physical barriers like waxy cuticle on the leaf surface, cell wall strengthening (Fu and Dong, 2013) and

callose production (Conrath, 2011), plants produce constitutive and/or inducible chemical defenses. For acclimation to changing environmental conditions such as temperature, light, drought or CO<sub>2</sub> level, land plants use endogenous signals, such as jasmonic acid (JA) and salicylic acid (SA) (Conrath et al., 2006). Against biotic stresses, plants defenses can act locally against the enemy, like the acacia tree which produces higher concentrations of cyanide at the site of grazing (Fu and Dong, 2013). Plants can also set up defenses remotely either systemically in the whole plant or by allelopathy where chemical compounds act on the neighboring plants by preventing them of a danger or by attracting herbivore enemies (Conrath, 2009). For example, the phytohormones ethylene, JA, SA and abscisic acid (ABA) are involved in distance signaling in plant-insect interactions, from roots to shoots (Soler et al., 2013). As abiotic and biotic stresses trigger degradation and oxidation of plasma membrane components, the release of free fatty acids and oxidized derivatives such as oxylipins is likely to be a key component of defense signaling processes (Mithöfer et al., 2004).

## 2. Perception of grazing stress and direct chemical defenses against herbivory

### a. Chemical recognition of biotic stress

Prerequisite to the induction of effective defenses is the plant perception of an external threat by the recognition of attack-derived molecules (Meng and Zhang, 2013). These molecules are grouped under the term of elicitor, which can be defined as a compound inducing defense responses in plants (Conrath et al., 2002). Elicitors can be of exogenous nature, generally termed Microbe-Associated Molecular Patterns (MAMPs), and defined as a set of molecules coming from the microorganism or the herbivore that are recognized by the plant host immune system. Alternatively, endogenous elicitors or Microbe-Induced Molecular Patterns (MIMPs), are derived from host compounds, such as host cell wall degradation products, through the action of the aggressor (Staal and Dixelius, 2007). MIMPs can be (poly)peptides, glycoproteins, lipids, oligosaccharides, whereas MAMPs are represented by lipopolysaccharides (LPS) from Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, eubacterial flagellin, methylated bacterial DNA fragments and fungal cell wall-derived glucans, chitins, mannans, or proteins (Nürnberg et al., 2004). While MAMPs are not part of the host which qualifies them as ‘non-self’, a MIMP may not contribute to pathogen virulence, but is rather a by-product of the herbivore or microbe’s action, constituting ‘self’ molecules (Mackey and McFall, 2006). These ‘self’ molecules can act as alarm signals, indicating a danger to the plant immune system. Their recognition by receptors thus initiates a signal transduction cascade, like calcium flux, activation of mitogen-activated protein kinases (MAPKs), resulting in early defense responses such as the massive and rapid production of reactive oxygen species (ROS) named the oxidative burst, as well as the induction of

ethylene biosynthesis. Furthermore, recognition of MAMPs and MIMPs is a common trait of innate immunity in animals and in plants (Mackey and McFall, 2006).

#### b. Local and intra-plant systemic defense signaling

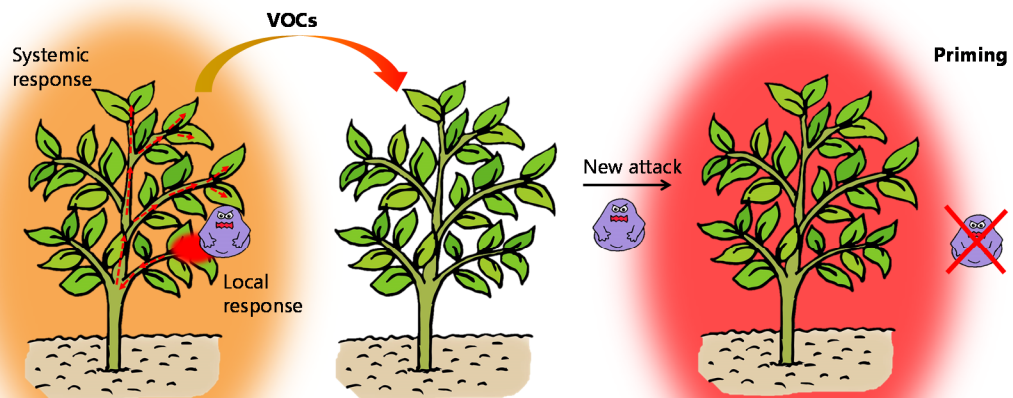
Besides constitutive anatomical and morphological traits, plants also respond to herbivores or pathogens by chemical inducible traits (Heil, 2010). These traits correspond to the production of chemical compounds, and their accumulation in plant tissues, generally in the attacked or infected tissues. Other compounds can affect digestibility of plants like proteinase inhibitors, or repellent compounds which can deter feeding by herbivores (Baldwin et al., 2001; Heil, 2010). For instance, jasmonic acid (JA) was shown to mediate direct defense responses against both chewing and sucking insects, as well as against the necrotic pathogens *Botrytis cinerea* and *Alternaria brassicicola* in *Arabidopsis* (Chehab et al., 2008). Glucosinolates produced in *Arabidopsis* and other plant species in response to herbivore attacks were shown to play a repellent role against generalist herbivores (Baldwin et al., 2001).

As herbivores are mobile, plants respond to attacks not only locally but also systemically in the whole plant, i.e. in healthy undamaged organs, limiting the spread of the attacker to the other parts of the plant (Heil and Ton, 2008; Heil and Karban, 2010). The Systemic Acquired Resistance (SAR) was first highlighted in tobacco, where inoculation of lower leaves with the tobacco mosaic virus (TMV) induced a higher resistance to a second infection in upper leaves (Ross, 1961). Then, SAR was described in many plants and was shown to be triggered by salicylic acid (SA) which acts as a signal transporter through the plant (Heil and Ton, 2008; Conrath, 2009). However, it was demonstrated that SA is not crucial for SAR induction (Heil and Ton, 2008). In addition, SA is known to inhibit the hypersensitive response when innate immunity is activated, to trigger temperature elevation in plants, and to inhibit plant growth, chloroplast development, and photosynthesis (Fu and Dong, 2013). Jasmonic acid (JA) is also involved in wounding-induced responses and is required in distal plant parts for a systemic induction of protein inhibitors, which act negatively on plant tissue digestion by herbivores. At the site of damage, linolenic acid from membranes is released and enzymatically transformed until production of jasmonates (see Part 3) (Feussner and Wasternack, 2002). Establishment of SAR requires the induction of some genes, such as those encoding pathogenesis-related proteins (PR-proteins), which have antimicrobial activity and play a dual role together with SA (Conrath et al., 2002; Conrath et al., 2006).

### 3. Long distance airborne signaling

#### a. Priming and plant-plant communication

In some studies, a resistance was observed in distal plant parts which lacked direct vascular connection with damaged parts, as quickly and strongly as in plant parts connected by the vascular system (Heil and Ton, 2008). This external within-plant signaling has now been demonstrated in sagebrush, lima bean, poplar and blueberry, where VOCs released from damaged plant tissues induced resistance in distant undamaged organs of the same plant. In this case, distant plant parts can perceive VOCs as warning signals, more rapidly than internal translocated signals, allowing fast establishment of resistance in the whole plant (Heil and Karban, 2010).

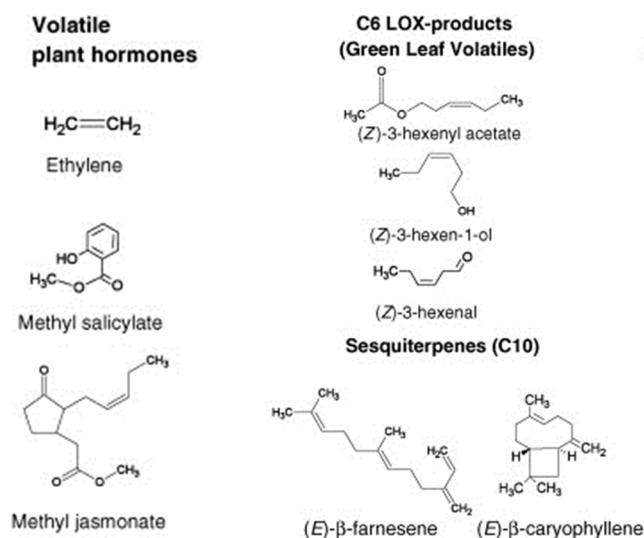


**Figure 2.** Representation of priming induction. The plant attacked by herbivores releases volatile organic compounds (VOCs) which are perceived by a neighboring plant. Only after a new attack, this second plant is able to defend more efficiently and rapidly to herbivores.

The first evidence of plant-plant communication was proposed by Baldwin and Schultz in 1983. They found that after damage of the poplar *Populus euroamericana* and the sugar maple *Acer saccharum*, damaged plants contained higher amounts of phenolic compounds than undamaged plants. They also demonstrated that their neighboring plants showed similar chemical changes and that these biochemical changes could influence the growth and feeding of herbivores (Baldwin and Schultz, 1983). First performed in laboratory, experiments on plant-plant communication were then made under natural conditions, which provided more evidence of the existence of airborne chemical cues and their impact on the resistance of the receiver organism (Beckers and Conrath, 2007; Heil and Karban, 2010). However, most of these studies were conducted in an ecological context and focused on anti-herbivore defense through plant communication, thus less concerning the volatile-mediated signaling and the perception of the signals by neighboring plants. Studies on plant-plant communication allowed to highlight a defense process in plants, known as ‘priming’ (Fig 2). The VOC-receiver primed plant does not undergo immediate and important phenotypic modifications, but develops a faster and stronger response upon a new herbivore attack (Heil and Karban, 2010). However, it appears that priming does

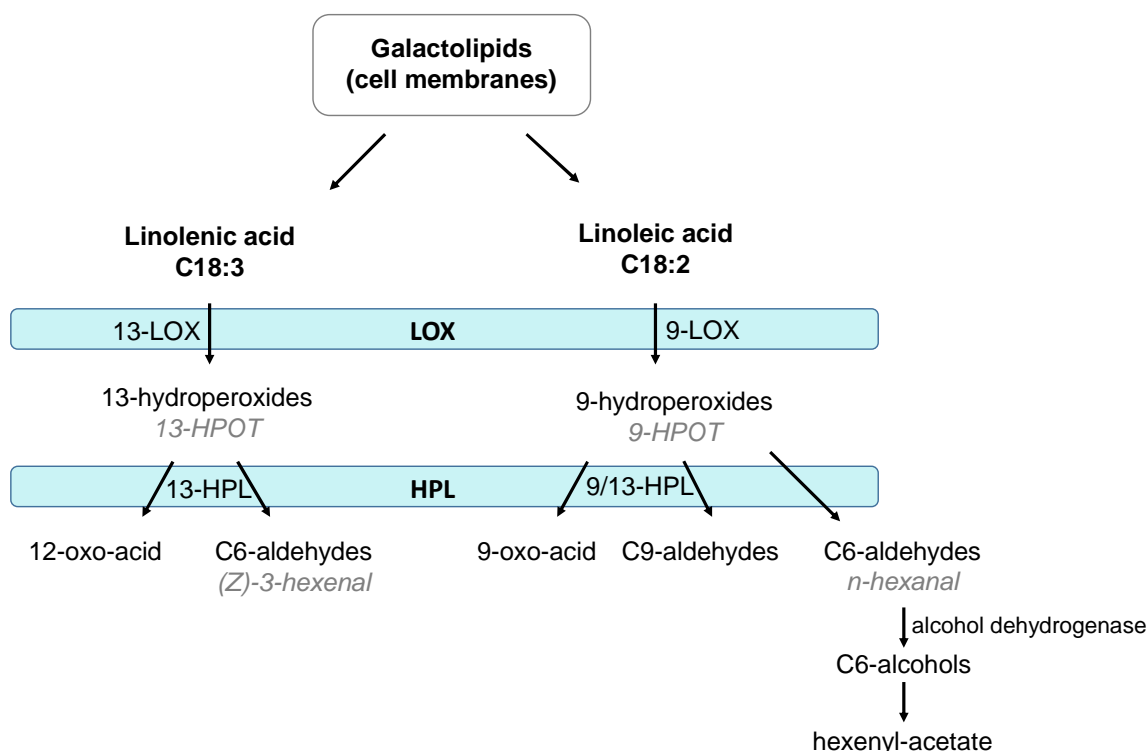
not induce resistance without confirmation of damage by SAR signaling (Heil and Ton, 2008) and requires modification of chromatin to establish histone memory and information storage for future plant stress responses (Jaskiewicz et al., 2011).

Different chemical volatile compounds were shown to be involved in plant-plant communication upon insect feeding, such as methyl jasmonate (MeJA), methyl salicylate (MeSA), ethylene and green leaf volatiles (GLV), which are C6 volatiles (Fig 3). Each of these compounds acts differently within a few minutes on plant physiology through gene activation and induces the production of various chemical compounds (Heil and Ton, 2008; Baldwin, 2010). After exposure of lima bean *Phaseolus lunatus* to the aldehyde nonanal for 6 h, its resistance to the bacterial pathogen *Pseudomonas syringae* was increased, whereas low concentrations of MeSA for 6 h did not reduce bacterial infections (Girón-Calva et al., 2012). Similarly,  $\beta$ -aminobutyric acid (BABA) emitted during plant stress was shown to induce resistance against a pathogen, in laboratory as well as in field experiments (Beckers and Conrath, 2007). For example, the application of this compound induces resistance in grape against the oomycete *Plasmopora viticola* and suppresses disease symptoms due to *Phytophthora infestans* in potato and tomato plants (Beckers and Conrath, 2007; Ton and Jakab, 2007). Moreover, some signal compounds produced after herbivore attack also induce plant protection against pathogens. For example, methyl jasmonate (MeJA) suppresses the growth of several plant pathogens and limonene stops the growth of the fungus *Fusarium verticillioides* (Heil, 2014). Jasmonate as well as other oxylipins such as epoxy-, hydroxyl-, and divinyl ether derivatives, which are derived from polyunsaturated fatty acids (PUFAs) hydroperoxidation, have also been described to be associated with plant defense responses against pathogens and herbivores (Feussner and Wasternack, 2002; Mithöfer et al., 2004).



**Figure 3.** Examples of plant volatiles involved in distance signaling in response to herbivores. Phytohormones are represented by ethylene, methyl salicylate (MeSA) and methyl jasmonate (MeJA). Among C6-LOX products, Green Leaf Volatiles (GLV) are part of the Volatile Organic Compounds, together with sesquiterpenes for instance.

GLVs comprise C6 volatile compounds such as aldehydes, alcohols and their esters, and are produced enzymatically by the LOX pathway (Arimura et al., 2009). They usually come from disruption of galactolipids composing cell membranes, providing free fatty acids such as linolenic and linoleic acids (Matsui, 2006). These fatty acids are transformed by a lipoxygenase (LOX) into hydroperoxides, either on position 13 or 9 of the fatty acid (Fig 4). When the hydroperoxide 13(S)-hydroperoxy-(Z,E,Z)-9,11,15-octadecatrienoic acid (13-HPOT) is formed from linolenic acid, it can then be metabolized by a 13-hydroperoxide lyase (13-HPL) to the aldehyde (Z)-3-hexenal and the oxo acid 12-oxo-(Z)-9-dodecenoic acid. From linoleic acid, the alkane n-hexanal is formed. Then, C6-alcohols can be formed by the action of an alcohol dehydrogenase and an acyltransferase allows the formation of (Z)-3-hexenyl acetate from (Z)-3-hexen-1-ol and acetyl CoA. When the 9-hydroperoxides are produced by 9-LOX, 9/13-HPL catalyze the formation of C9-aldehydes and C9-oxo acids (Matsui, 2006). It has been shown that the aldehyde (Z)-3-hexenal was emitted in the vicinity of wounded sites of *Arabidopsis* immediately after injury, followed by (Z)-3-hexen-1-ol and hexenyl acetate in lower amounts (Arimura et al., 2009). Direct effects of GLVs were observed on microorganisms and it seems that their bactericidal activity is due to their chemical structure: the  $\alpha,\beta$ -unsaturated carbonyl moiety is highly reactive with nucleophiles through Michael-type addition reactions (Matsui, 2006).



**Figure 4.** The LOX pathway in plants producing Green Leaf Volatiles. From galactolipids provided by cell membranes, linoleic (C18:2) and linolenic (C18:3) acids give rise to hydroperoxides and then aldehydes through enzymatic transformations. Then, alcohols and hexenyl acetate are derived from aldehydes by the action of an alcohol dehydrogenase and an acyltransferase, respectively. LOX: lipoxygenase; HPL: hydroperoxyde lyase; HPOT: hydroperoxyoctadecatrienoic acid.

A priming effect was also demonstrated in bean plants, when application of nonpathogenic *Pseudomonas putida* reduced disease symptoms induced by pathogenic *Botrytis cinerea*, after higher production of GLVs. Their protective effects were also shown against plant fungal pathogens in *Arabidopsis* and in *Citrus*, and against herbivores in corn through activation of priming (Engelberth and Alborn, 2004). Prior exposure of corn to these compounds induce production of JA and VOCs after elicitation, leading to inducible direct and indirect defense responses. Moreover, it was shown that GLVs emitted by beetle-damaged lima bean plants reduced herbivore damage and increased the growth rate of neighboring uninfested conspecifics (Heil and Silva Bueno, 2007). GLV emitted by lima bean also induce pathogenesis-related protein transcriptional responses, involved in the onset of SAR but they are no longer considered as essential in plant resistance (Baldwin et al., 2002; Conrath et al., 2006; Conrath, 2009). Thus, GLVs can be carried out by air from a plant to another one, even at low concentrations, and can promote defense resistance in receiving plants, acting as priming signals, or inducing SAR-related defense mechanisms when they act as exogenous elicitors (Matsui, 2006).

#### b. Green leaf volatiles and multitrophic interactions

In natural environment, GLVs are also perceived by other insects in the surroundings of the emitting plant, leading to multitrophic interactions. The VOCs emitted by plants under herbivore attack can have a protective effect as they may provide information representing a 'cry for help' for the plant (Heil, 2008). Indeed, natural enemies of herbivores like carnivorous insects or parasitic wasps, are effectively attracted by these VOCs, thus serving as an additional means of plant resistance. The first study which demonstrated that herbivore-induced volatiles attract herbivore enemies in natural communities was performed by Kessler and Baldwin in 2001. They showed that, upon herbivore attack, *Nicotiana attenuata* released a complex blend which attracted herbivore predators. Among them, cis-3-hexen-1-ol, linalool, and cis- $\alpha$ -bergamotene increased egg predation rates and the complex blend could decrease the oviposition rates (Kessler and Baldwin, 2001). Later, it was found that previous exposure of lima bean plants to VOCs before mechanical damage had a priming effect, leading to secretion of extrafloral nectar (EFN) which plays a role in herbivore predators' attraction (Heil and Kost, 2006; Kost and Heil, 2006). The chemical signature of emitted GLV differs according to the specific interaction between the herbivore and its host. Carnivores can thus distinguish damaged from undamaged plants, as well as different herbivores attacking a plant, according to the blend of compounds emitted by the damaged plant. Moreover, it seems that the type of feeding damage affects the types of VOCs produced. Hence, leaf chewers generally induce jasmonic acid (JA) signaling, while piercing-sucking herbivores (phloem feeders and single-cell feeders) induce resistance via salicylic acid (SA) pathway (Heil, 2008). These precise metabolic regulations allow the plant to finely adjust its defense mechanisms according to the needs, which is also an advantage in terms of energy costs.

### c. Costs and benefits of distance airborne signaling

As plant herbivores can easily move from one leaf to the neighboring one, a physiological obvious advantage of airborne signaling compared to SAR is the independency from the plant's vascular system. A fast signaling is crucial for defense against herbivores, within the same plant or among neighboring conspecifics. Nevertheless, both distance signaling, SAR and airborne communication, act synergistically against enemies to achieve an optimized orchestration of the defense response (Heil and Ton, 2008). Furthermore, responding to volatile cues has been shown to increase components of receiver fitness by a higher resistance to pathogens and herbivores (Heil and Karban, 2010). However, receiving a chemical message could be seen as 'eavesdropping' rather than a true communication, and emission of volatile compounds could induce metabolic costs through their production and emission processes. Thus, in this case, neighboring plants fitness is increased without enhancing fitness of the emitting plant and this could lead to an evolutionary disadvantage of the emitter. Evolutionary theory predicts that fitness costs can be compensated by fitness gains of neighbors if these are actually close relatives. In this case, fitness of a genotype providing benefits to its sibs and other close relatives include the fitness of these relatives as they all share a large number of alleles throughout the genome, alleles that are favored thanks to the costly action of the focal genotype (Maynard-Smith, 1964). This theory has been developed and tested mostly in eusocial animals, but may explain how the release of signals for the benefit of neighbor plants has evolved besides the evident cost to the emitting plant.

Other costs include the use of these VOCs by herbivores or parasites for host localization (Heil and Karban, 2010). More than one species of herbivore enemies can also be attracted by these VOCs, or too many individuals of the same species, leading to competition and thus decreasing the positive effects of the intervention of herbivore enemies. The metabolic balance between growth or reproduction and defense can also be affected, as resources used for defense could not be allocated for primary functions (Conrath, 2009; Unsicker et al., 2009). Nevertheless, after perception of the chemical message, neighboring plants can adjust their physiology accordingly, allowing investment of metabolic resources only when defenses are needed. In a population level viewpoint, helping neighbors is considered to be favored and more stable in populations with limited dispersal, as a high proportion of them comes from the direct offspring of the emitting plant. Thus, warning neighbors could increase the fitness of an individual, through a positive effect on the survival of its offspring (Heil and Karban, 2010). Moreover, the intervention of a third trophic level during multitrophic interactions provides an obvious advantage to the plant which attracts predators of herbivores, but also for the predators which rely on VOCs to locate their feeding source (Heil, 2008).



## II. Chemical mediation in the marine environment

### 1. Marine chemical ecology

#### a. Algal chemical ecology

Research in marine chemical ecology includes investigations on a wide diversity of marine organisms such as microorganisms (bacteria, viruses, fungi), macroalgae, sponges, cnidarians, crustaceans, molluscs, fish or corals, and covers a large range of interactions that organisms have with their biotic and abiotic environments (Paul et al., 2011; Amsler, 2012). This research domain can be divided into sensory and defensive chemical ecology. Sensory chemical ecology concerns chemical communication between organisms, as well as perception and response to the abiotic environment. Chemical communication is essential in processes of brown alga's sexual reproduction, as pheromones released by female gametes attract male gametes and sometimes stimulate the release of male gametes (Maier and Müller, 1986; Pohnert and Boland, 2002). This was demonstrated in the brown alga *Ectocarpus siliculosus* for which the C11 pheromones ectocarpene and pre-ectocarpene secreted by the female gamete induce a modification in the male gametes movements, then trapped in the female gamete area until they are in contact (Müller, 1967). In *Laminaria digitata*, the C11 pheromone lamoxirene released by the female gametes attracts and also stimulates the release of male gametes (Maier and Müller, 1986). Perception of the abiotic environment was shown to induce responses in spores of brown algae, in particular in several species of kelps like *Laminaria japonica*, whose swimming behavior varies according to nutrients, subsequently used for gametophyte development (Fukuhara et al., 2002). Besides sensory chemical ecology, researchers also investigate defensive processes involved in interactions between organisms such as predation, biofouling, competition and defense against pathogens (Amsler, 2012). Algal defenses can be localized in some parts of the thallus and also diffused throughout and outside the seaweed (Jormalainen and Honkanen, 2008). Several studies have focused on defense against predation in brown macroalgae by means of phlorotannins (Pavia and Toth, 2000; Amsler and Fairhead, 2006), while investigations on green algal defense allowed the identification of, for example, caulerpenyne from *Caulerpa sp.* and dimethylsulfoniopropionate (DMSP) from *Ulva* and related genera, as feeding deterrent against some herbivores (Jung and Pohnert, 2001; Van Alstyne, 2008). Caulerpenyne was also found to be converted in highly reactive aldehydes, potentially having a defensive role against herbivores. Algal defense against pathogens include production of chemical compounds against bacteria, fungi and filamentous endophytes (Amsler, 2012). Reactive oxygen species at sufficient concentrations as well as organic compounds were found to inhibit their growth and contain the infection. Against biofoulers, these chemical compounds have to be also present at the algal surface at biologically effective concentrations (Steinberg and De Nys, 2002). This is the case of the red alga *Delisea pulchra* which produces halogenated furanones at sufficient concentrations on its surface to act

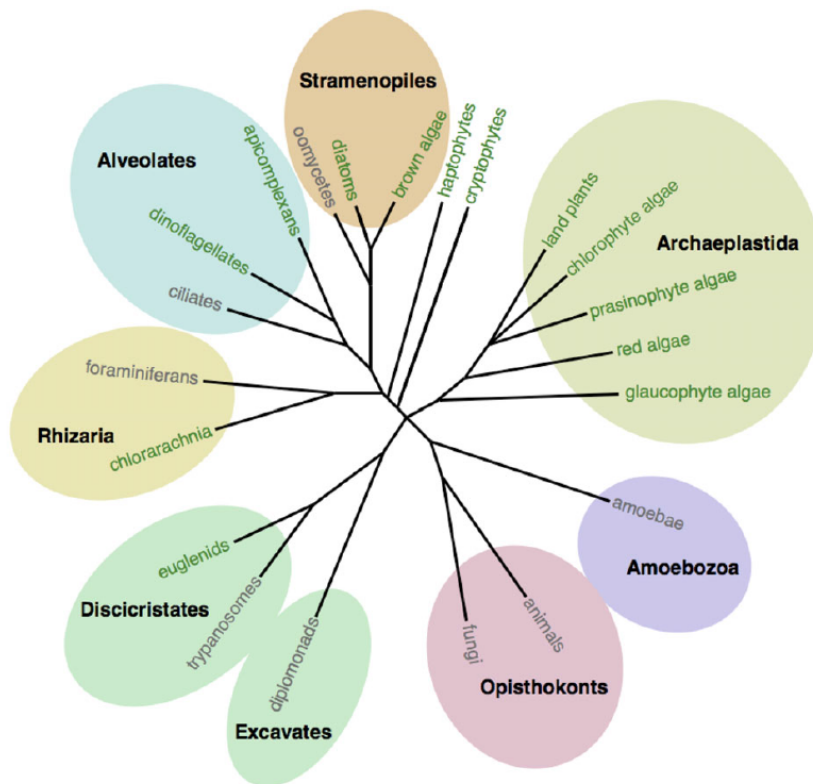
against predators and biofoulers (Dworjanyn et al., 1999). Halogenated furanones compete with and inhibit quorum sensing communication among bacteria, thus preventing from biofilm formation (Harder et al., 2012). Finally, defense against competitors in the marine environment is represented by allelopathy, which has already been described between the red alga *Plocamium hamatum* and corals, causing tissue necrosis in soft corals with the monoterpene chloromertensene, or bleaching by several algal species (Amsler, 2012). Moreover, these effects were correlated with the presence of lipophilic chemical compounds in algal extracts, caused by the physiological stress of the microalgal symbionts.

#### b. Characteristics of the marine environment

Thus, similarly to land plant chemical ecology, algal chemical ecology covers a large range of biological roles in different types of interactions, involving various and numerous chemical compounds. The marine environment, however, presents some unique characteristics in terms of physical and ecological properties which impact the chemical mediation in the marine medium.

##### - Evolutionary aspects

Marine algae belong to different independent eukaryotic lineages. Chlorophytes (green algae) and Rhodophytes (red algae) are sister groups in the same lineage leading to land plants, called Plantae or Archeplastida, while Phaeophytes (brown algae) evolved independently into the Stramenopiles or Heterokonts lineage (Fig 5). Brown algae are evolutionarily close to diatoms, which also belong to the Stramenopiles group. The emergence of these different macroalgal groups is linked to the endosymbiosis origins of their plastids. First, a heterotrophic eukaryotic cell has incorporated a photosynthetic ancestral cyanobacteria, giving rise to the first photosynthetic ancestral Eukaryote, at the origin of Chlorophytes and Rhodophytes. Later, during evolution of Eukaryotes, second endosymbiosis events, corresponding to the endocytosis of an autotrophic unicellular eukaryote by a heterotrophic eukaryote would have occurred several times independently, to give rise to the actual diversity of marine photosynthetic organisms. The Stramenopiles, and thus the Phaeophytes, derived from the secondary endosymbiosis of an ancestral unicellular red alga by a heterotrophic protist. Thus, this evolutionary context in Eukaryotes suggests that some traits of the chemical communication, for instance regarding the biosynthetic pathways or identity of chemical compounds, in brown algae could be shared with other photosynthetic Eukaryotes, while other traits could be specific of the brown algal lineage, like for iodine emission or phlorotannins production.



**Figure 5.** Phylogenetic tree of Eukaryotes and evolution of Brown algae among Eukaryotes.

Photosynthetic organisms such as macroalgae are the unique source of  $\omega$ -3 and  $\omega$ -6 essential fatty acids for upper trophic levels, and their fatty acid composition is consistent with these evolutionary processes. Whereas similarities exist between red and brown algae in term of polyunsaturated aldehydes (PUAs) composition, the brown algal lineage could have evolved distinct biochemical pathways in fatty acid metabolism, which could possibly have specific defensive effects on their herbivores. Contents in 44 fatty acids were recently investigated in 40 taxa of green, red and brown macroalgae, and it was found that this composition differed significantly according to the phylum, order and family of the taxa (Galloway et al., 2012). The red and brown algae mostly contain the fatty acids arachidonic acid (ARA, C20:4  $\omega$ -6) and eicosapentaenoic acid (EPA, C20:5  $\omega$ -3), whereas red algae contain more C16:0 and C18:0 than brown algae. However, green algae are mostly composed of linoleic acid (LIN, C18:2  $\omega$ -6), linolenic acid (ALA, C18:3  $\omega$ -3) and stearidonic acid (SDA, C18:4  $\omega$ -3).

- Physical characteristics

The marine environment is defined by physicochemical characteristics which differ from those encountered in the terrestrial environment, mainly because the environment is liquid water, thus modifying some aspects of the chemical communication between organisms. Indeed, the carrier of the information is mostly seawater and occasionally air, depending on the amplitude of the tide, thus

potentially involving soluble as well as volatile molecules. In addition, these compounds are often composed of halogenated elements such as iodine and bromine (Hay and Fenical, 1988).

- Ecological aspects

Marine ecosystems provide the vast majority of the primary production of the Earth, through the plankton. In coastal environment, macroalgae are the main primary producers. It was demonstrated that the abundance and the growth of the primary producers were strongly affected by grazing by herbivores, even in low rates (Poore et al., 2012; Poore et al., 2014). The marine and terrestrial environments can also be distinguished by a difference in abundance of specialist herbivore communities, barely represented in the marine environment (Hay and Steinberg, 1992). Indeed, most marine herbivores (such as fishes, sea urchins, amphipods, crabs, polychaetes and gastropods) are feeding generalists, capable of feeding on 10 to 20 families of seaweeds. Specialist herbivores exist in marine communities, but they are in limited amounts (Hay and Steinberg, 1992). Grazing by invertebrate mesograzers such as amphipods, polychaetes, small crabs, and gastropods usually removes a small amount of algal production (<10%) (Carpenter, 1986). Thus, defense evolution against marine herbivores is directed primarily by generalist herbivores of low to moderate impact, and it was proposed, according to Hay and Fenical (1988), that the potential for coevolution between a plant and its herbivore is low in marine communities.

Yet, marine algae do not possess a vascular system like in plants that allows transportation of signals towards distant areas from their production site (Cronin and Hay, 1996c). These systems provide a distance communication into the organism and allow the establishment of the systemic acquired resistance (SAR) (Heil and Ton, 2008; Shah, 2009). Multicellular marine algae, however, have evolved analogous systems for intra-plant communications for effective SAR-like defenses against herbivores. Translocation of different soluble compounds by communication between adjacent cells have been demonstrated in very large algae, such as kelps (Floc'h and Penot, 1978) and recent results based on the oligoguluronate-induced defense responses suggested the occurrence of a systemic protection against herbivores in *L. digitata* (Thomas et al., 2014). Seaweeds are also capable of producing a wide range of secondary metabolites, such as terpenes, aromatic compounds, amino acid-derived substances, and phloroglucinol-based polyphenolics, which could play roles in inter-plant defense signaling (Hay and Steinberg, 1992). But if the nature of volatile compounds (e.g. their volatility) depends mostly on molecular weight and temperature in airborne environment, the important factor in water is their diffusion rate (Steinke et al., 2002). Thus, when releasing complex blends of compounds, algae can create a chemical gradient in their surroundings with different physical, chemical and likely signaling potentials. Finally, the efficacy of a chemical signal depends on its recognition by the receiver (Steinke et al., 2002).

## 2. Chemical mediation in micro- and macroalgae in response to grazing stress

### a. Chemical defense in diatoms against copepods

Even if diatoms were previously considered as an optimal food for copepods, the first evidence of inhibitory effects of feeding by diatoms on hatching success of copepods demonstrated that the diatom *Thalassiosira rotula* had a negative effect on *Temora stylifera* egg viability (Ianora and Poulet, 1993) and later, that antimetabolic compounds of this diatom species blocked embryogenesis of the copepod *Calanus helgolandicus* (Poulet et al., 1994). Other negative effects were highlighted on generation time and mortality rates of these grazers (Ianora et al., 2003). The diatom-copepod model has been extensively studied and according to (Ianora and Miralto, 2010), it turns out that it has no equivalent in marine plant-herbivore systems. Indeed, most prey-predator chemical defense interactions in the marine environment are based on repellency or poisoning, whereas diatoms are exclusively effective on predator's reproductive failure. D'Ippolito et al. (2002) found that, when fed with the diatom *Skeletonema costatum*, egg production rates and hatching viability of the copepod *Temora stylifera* were strongly affected by food. Hatching success even decreased to 0% within 7 days after feeding with *S. costatum*. This effect was also tested with the diatom species *Thalassiosira rotula*, but it was less intense as it did not modify egg production rates though decreasing hatching success (D'Ippolito et al., 2002b). However, when this diatom was used as food for the copepod *Calanus helgolandicus*, hatching success was strongly reduced. Development of embryos was abnormal, with dispersed chromatin and asymmetrical division of blastomeres, preventing from egg hatching (Ianora et al., 2003). Moreover, inhibitory effects are diatom density-dependent, high diatom densities triggering less hatching success than low densities. In 1999, Miralto et al. observed a significant decrease of hatching success in copepods and a negative effect on cleavage in sea urchin embryos feeding on different diatom species and found that three aldehydes isolated from the diatoms were responsible for this biological activity: 2,4-decadienal and 2,4,7-decatrienals. However, it seems that inhibitory activity was not limited to those aldehydes, but concerned a major part of the poly-unsaturated aldehydes (PUAs), containing  $\alpha,\beta,\gamma,\delta$ -unsaturations, excluding aldehydes without unsaturations which do not have effects on predators (Pohnert et al., 2002; Pohnert, 2004). Active extracts of *S. costatum* against copepods were shown to contain trans,trans-2,4-heptadienal, trans,cis-2,4-octadienal, trans,trans-2,4-octadienal, and 2-trans-4-trans-2,4,7-octatrienal. These authors also found the latter two compounds in the diatom extracts of *Thalassiosira rotula* after sonication (D'Ippolito et al., 2002a). Other studies showed inhibitory effects of different species of diatoms on egg-hatching success and on copepod fecundity, but it appears that not all copepods were equally sensitive to diatom metabolites, and that diatom do not induce the same negative effects on copepods (Ianora et al., 2003). Therefore, investigations increased on the roles and characterization of these PUAs in defense of diatom species against other marine organisms, such as sea

urchin embryos, polychaetes and sea star embryos. Both diatom extracts and the aldehyde decadienal suppress fertilization, embryogenesis and hatching success of these organisms (Pohnert, 2005). Moreover, decadienal was found to significantly reduce hatching success of polychaete at 125 ng.mL<sup>-1</sup>. However, reactive aldehydes were also shown to be involved in communication between diatoms, as both growth and cell cycle of the diatom *Thalassiosira weissflogii* were influenced by the aldehyde 2-trans,4-trans-decadienal (Ianora et al., 2003). Thus, this is well established that PUAs present in diatoms play a crucial role in regulation of copepod communities, depending on diatom and copepod species, in the laboratory. Laboratory experiments also demonstrated that the PUAs 2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal inhibited the growth of two bacterial strains associated with diatom species *Skeletonema marinoi* in the Northern Adriatic Sea (Ribalet et al., 2008). However, attempts of application of these experiments in higher scales to try to come closer to *in situ* conditions were not as conclusive as laboratory experiments. Paul et al. (2012) tested the roles of PUAs in bacteria-diatom interactions as chemical mediators in mesocosm conditions. They found no effect of these PUAs on bacterial and viral abundance, as well as on bacterial community composition, but rather an alternation of bacterial diversity over time and a diatom strain-dependent bacterial diversity. The authors concluded that PUAs did not significantly affect bacteria-diatom interactions, even though they could influence interactions with other marine organisms (Paul et al., 2012).

#### b. Defensive strategies in macroalgae against herbivory

In response to grazing by herbivores, macroalgae simultaneously employ different strategies in order to defend themselves and conserve high fitness. They use constitutive defenses, that are constant defenses at effective levels, as well as inducible defenses which spread out only in response to a cue indicating a higher risk of grazing (Jormalainen and Honkanen, 2008). The environmental conditions in which macroalgae live dictate the type of activated defenses, without exclusivity. There can be a certain level of constitutive defense and, if needed, inducible defenses can rapidly rise for higher protection of the alga. Though, constitutive and inducible defenses have not the same ratio in costs/benefits balance: since constitutive defenses are constantly produced, they require higher energy costs than inducible defenses which are only activated in response to a warning cue. Marine grazers are considered as feeding over spatial and temporal scales allowing preferentially inducible to constitutive defenses (Jormalainen and Honkanen, 2008). This hypothesis is more and more supported, as observations of inducible defenses with direct anti-grazing effects are accumulating for marine macroalgal species (Cronin and Hay, 1996a; Pavia and Toth, 2000; Sotka and Hay, 2002; Taylor et al., 2002; Toth et al., 2005).

In the red algae *Gracilaria vermiculophylla* and *G. chilensis*, grazing by the sea snail *Echinolittorina peruviana* induces chemical defenses, making them less attractive for a subsequent grazing (Rempt et al., 2012). Moreover, it appears that the oxylipins 8-hydroxy eicosatetraenoic acid (8-HETE) and 7,8-dihydroxyeicosatetraenoic acid (7,8-di-HETE) are released by these macroalgae and

that they have inhibitory effects on the development of other red algae (Lion et al., 2006). 7,8-di-HETE was also found to be active against the herbivore *Echinolittorina peruviana*, while prostaglandins and other oxylipins do not have inhibitory effects (Rempt et al., 2012). In the brown alga *Dictyota menstrualis*, Cronin and Hay (1996) demonstrated that grazing by the generalist amphipod *Ampithoe longimana* increased amounts of defensive compounds in the alga, reducing the susceptibility of the alga to be subsequently grazed by the amphipod. In particular, metabolomics analyses of algal tissues complemented by choice and non-choice bioassays allowed the identification of three diterpenoid secondary metabolites produced in damaged plants - dictyol E, dictyodial and pachydictyol A – which decreased consumption by fish, sea urchins and the amphipod *Ampithoe longimana*. For example, in Dictyotales species, pachydictyol A was fed to the omnivorous fish *Diplodus holbrooki* and it resulted in a significant decrease of their growth rate, compared to control fish that consumed equivalent amounts of food without the compound (Hay et al., 1988). The inhibitory effect of these compounds was different according to the herbivore species and their localization into the algal thallus (Cronin and Hay, 1996b). Similar inducible inhibitory effects were observed in the brown alga *Fucus vesiculosus*, where *Idotea baltica* herbivores preferred control algae over grazed plants (Haavisto et al., 2010; Yun et al., 2010) whereas *L. littorea* did not have preferences for control or grazed *F. vesiculosus* plantlets (Yun et al., 2010). Interestingly, mechanical damage did not induce algal resistance (Rohde et al., 2004) but, on the contrary, increased algal consumption by *I. baltica* (Haavisto et al., 2010). This may come from the way of wounding, allowing better digestibility of algal pieces. In the kelp *Laminaria japonica*, grazing by *Littorina brevicula* reduced the palatability of algae for a subsequent grazing, but this effect was not observed after grazing by the abalone *Haliotis discus* (Molis et al., 2008). This experiment was also made on *Ulva pertusa*, but did not show specificity of grazer species. Direct grazing effects were also observed in two other brown algal species, *Ascophyllum nodosum* (Pavia and Toth, 2000) and *Fucus distichus* (Van Alstyne, 1988). In response to a direct grazing by its specialist herbivore *Littorina obtusata*, *A. nodosum* produces phlorotannins within a few weeks. Interestingly, this defense reaction was not found after grazing by the isopod *Idotea granulosa* or after a mechanical damage. Thus, there is a high degree of specificity in the chemical inducible responses, according to the physical or biotic damage applied to *A. nodosum* (Pavia and Toth, 2000) and *F. vesiculosus* (Rohde et al., 2004). The same was observed in *Fucus distichus*, where grazing by the snail *Littorina sitkana* triggers an increase of phlorotannins in algal tissues, but it appeared that mechanical damage also triggered an increase of ~20% of these defense compounds within two weeks (Van Alstyne, 1988). Highly damaged fronds of *A. nodosum* contained high concentrations of phlorotannins and grazed fronds contained more than 100% more phlorotannins than undamaged fronds. It was also found that damaged algae by *L. obtusata* were less susceptible to a subsequent grazing than undamaged algae, which is not the case after grazing by *I. granulosa*. When herbivores could choose between grazed and ungrazed *A. nodosum*, *L. obtusata* chose preferentially undamaged algae with lower concentrations of phlorotannins, whereas no differences were found with the grazer *I. granulosa* (Pavia and Toth, 2000). In *F. distichus*, wounded plants were

preferred by the snail *L. sitkana* to uninjured plants directly after damage, but herbivore choices changed two weeks after injury, at the time concentrations of phlorotannins increased in damaged algae (Van Alstyne, 1988).

Nevertheless, the roles of phlorotannins in induced defenses of macroalgae do not seem as clear as it was previously supposed. It appears that increases in phlorotannins after algal challenging vary over spatial and temporal scales and that their repellent effects depend on both host and herbivore species (Haavisto et al., 2010). Some studies did not find any induction of phlorotannins production upon natural (Long et al., 2007; Jormalainen and Honkanen, 2008) or mechanical wounding. Other studies found increased amounts of phlorotannin after physical and natural damage (Haavisto et al., 2010), but these authors demonstrated that the sole presence of herbivores induced the same amounts of these compounds without decreasing algal palatability. Thus, they concluded that phlorotannins were not induced by the defense response of the alga to herbivory, or at least that this response was not the only defense response which could explain the repellent effect of *Fucus vesiculosus* against the isopod *Idotea baltica*. Besides inhibitory effects of direct grazing on this alga, these authors found that herbivores consumed less algal pieces when it was first co-incubated with a previously grazed conspecific. Moreover, large females feeding on these treated algae had a lower reproduction success, that is fewer eggs laid and smaller total egg mass (Haavisto et al., 2010). Thus, indirect defenses against grazers seems to be a good strategy for algal defense, through chemical mediation between algae, by means of waterborne compounds which can be perceived by neighboring algae for prevention of a danger. Perception of warning signals trigger epigenetic modifications in the receiver as preparation for future resistance expression (Goellner and Conrath, 2008; Heil, 2010; Pastor et al., 2013). This type of defense was shown earlier in the knotted wrack *Ascophyllum nodosum*, a brown alga, and its specialist grazer flat periwinkle *Littorina obtusata* (Toth and Pavia, 2000). After incubation in seawater containing grazed *A. nodosum*, unharmed neighboring algae contained higher amounts of polyphenolic secondary metabolites, phlorotannins. Thus, grazing on *A. nodosum* induces the production of waterborne compounds which can serve as signals to induce production of secondary metabolites in their undamaged neighboring conspecifics. Moreover, the increasing levels of defense chemicals into algae which received the chemical signals prevent herbivores for a subsequent grazing (Toth and Pavia, 2000). This study proved that chemical signals are emitted by *A. nodosum* during grazing by its specific herbivore, and that these compounds are perceived by neighboring algae. Thereafter, this latter alga produces defense chemicals which enable the deterrence of herbivores for a subsequent grazing. In the species *Fucus vesiculosus*, both direct grazing and co-incubation with a grazed conspecific induce defenses against the same isopod (Rohde et al., 2004). Interestingly, if grazing by the isopod *Idotea baltica* triggers an induction of chemical defenses in the red macroalgae *Furcellaria lumbricalis*, *Delesseria sanguinea*, *Phyllophora pseudoceranooides* and in the brown algae *Fucus serratus* and *Fucus evanescens*, direct grazing on these algae does not induce defenses in their neighboring conspecifics, as co-incubated algae were as preferred



as their control (Rohde and Wahl, 2008a). Thus, it appears that induction of defense chemical compounds depends not only on the grazer species, but also on the algal host.

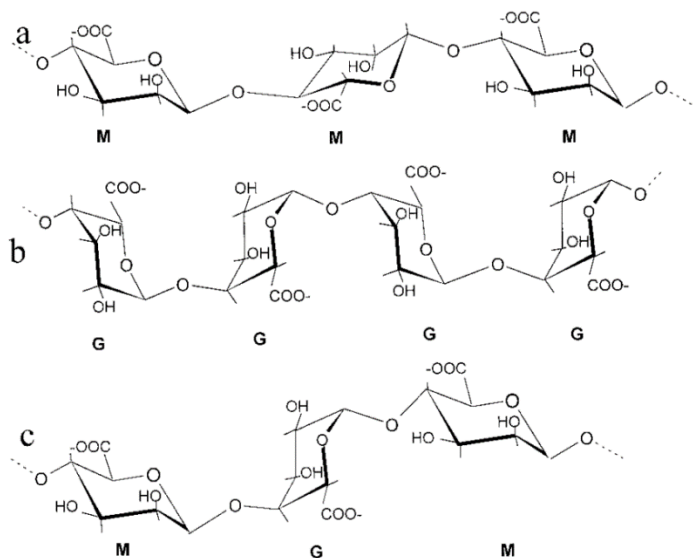
Indirect defenses include the attraction of other organisms as well, like predators of herbivores, via waterborne cues induced by algal responses. Involving different trophic levels, these interactions are defined as multitrophic. Some cases have been highlighted in the marine environment, like in the brown alga *A. nodosum* (Coleman et al., 2007). When this alga was grazed by its specialist herbivore *L. obtusata*, fishes were more attracted by its odours than by naïve or mechanically damaged plants, and crabs were also more attracted by these grazed algae than by naïve ones. Thus, grazed *A. nodosum* seem to release a specific bouquet which is perceived by predators of herbivores as food source, hence attracting them for herbivore consumption. The fact that specific blends are emitted by grazed algae, then perceived by predators could lead to evolutionary events like coevolution between these carnivores and the host algae. However, this remains to be demonstrated, as reciprocal responses among phenotypically plastic partners is a necessary prerequisite for the evolutionary stabilisation of mutualisms in general and, therefore, of indirect defense strategies.

### III. Innate immunity in algal lineages

#### 1. Recognition of MAMPs and MIMPs

Inducible algal defenses against pathogens and herbivores imposes activation of the defensive responses fast enough to limit the spread of the attacker before irreversible damage (Weinberger, 2007). This induction is based on molecular recognition of pathogen infections by innate receptors, located in the plasma membrane, for which some have already been characterized in metazoans and vascular plants (Schwessinger and Ronald, 2012). These innate receptors constitutively present in algae also allow distinction between self (or MIMPs) and non-self (or MAMPs). As previously described, MIMPs and MAMPs perception is followed by defense reactions in land plants. MAMPs are generally highly conserved between organisms and perceived by many eukaryotes (Nürnberg et al., 2004; Weinberger, 2007). In marine algae, MIMPs and MAMPs and their effects on algal resistance against pathogens have already been described in red and in brown algal lineages. For example in *Chondrus crispus*, resistance against its endophytic algal pathogen *Acrochaete operculata* was increased when the seaweed was treated with cell-free extract from this endophyte, considered as MAMPs (Bouarab et al., 2004). Similarly, MIMPs which can be degradation products of algal cell wall, for example fragments of alginates in brown algae or agar in red algae, having biological and ecological effects. In the red alga *Gracilaria chilensis*, agar oligosaccharides from algal cell wall increased the resistance of the seaweed against the epiphytic red alga *Acrochaetium sp.* (Weinberger, 2007).

In brown algae, products derived from alginates, the main brown algal cell wall polysaccharide, are one of the most studied MIMPs. They are composed of two types of linear polymers:  $\beta$ -1,4-D-mannuronic acid (oligomannuronates) and its C5 epimer,  $\alpha$ -1,4-L-guluronic acid (oligoguluronates) (Küpper et al., 2001). These oligosaccharides are arranged by alternation of homopolymeric blocks of poly- $\beta$ -1,4-D-mannuronic acid (MM blocks), of homopolymeric blocks of poly- $\alpha$ -1,4-L-guluronic acid (GG blocks), and of heteropolymeric blocks with random arrangements of both monomers (MG blocks) (Fig 6). Oligoguluronates are the functional analog of pectins in plants and agar in red algae and, like pectins, they form intermolecular complexes in the presence of calcium ions (Potin et al., 2002; Weinberger, 2007). The genome of the brown alga *Ectocarpus siliculosus* was mined in order to find homologues of genes encoding ligand-binding and signal transduction domains in plants and animals. It was found that the ROCO and NB-ARC-TPR families could be potential candidates for being involved in recognition/transduction events linked to immunity in the brown algal lineage (Zambounis et al., 2012). Studies on infection of *L. digitata* by its natural endophytic pathogen *Laminariocolax tomentosoides* demonstrated that previous elicitation by oligoguluronates was able to protect the kelp from infection by the endophyte within 7 days, compared to control kelps which were not previously elicited (Küpper et al., 2002). Epiphytic and endophytic filaments were not visible after elicitation treatment. In the kelp *Laminaria japonica*, infection with alginate-degrading bacteria have been shown to induce early responses similar to those following oligoguluronate application, such as the oxidative burst (Weinberger, 2007).



**Figure 6.** Chemical structure of alginate blocks from brown algae. Oligomannuronates (MM blocks; a) are linear arrangements of  $\beta$ -1,4-D-mannuronic acid, oligoguluronates (GG blocks; b) are formed with homopolymeric blocks of  $\alpha$ -1,4-L-guluronic acid and heteropolymeric blocks are composed of both monomers (MG blocks; c). From Küpper et al. (2001)

## 2. The oxidative burst

The oxidative burst is an important conserved component of innate immunity among all eukaryotes, from red and brown algae to vascular plants, as well as for animals (McDowell et al., 2014). It consists in a transient production of ROS, such as superoxide ions ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), or hydroxyl radicals ( $OH^{\bullet}$ ), through the activation of plasma membrane-associated NADPH oxidases or apoplastic peroxidases (Küpper et al., 2002; Weinberger, 2007). In the red macroalga *Gracilaria conferta*, an oxidative burst was observed after perception of products of the microbial degradation of its agar cell wall, thus protecting the alga against their epiphytic bacteria (Weinberger et al., 1999; Weinberger and Friedlander, 2000). In brown algae, Küpper et al. (2001) showed that oligoguluronate treatment on *Laminaria digitata* induced a high increase in oxygen consumption during 2 – 3 minutes after their addition. At the same time, the kelp releases a strong and sudden amount of hydrogen peroxide  $H_2O_2$  in the medium, reaching its maximum at 5 to 10 minutes after oligosaccharide addition, then decreasing until its basal level (Küpper et al., 2001). The induction of respiratory and oxidative bursts is dependent on the chemical structure of the oligosaccharides, GG blocks triggering them, while MM and MG blocks were not able to induce significant oxidative bursts. Moreover,  $H_2O_2$  emission rates and amplitude were also dependent on the concentration of alginate oligosaccharides applied to the kelp. An oxidative burst is observed with GG concentration ranging from  $2.5 \mu\text{g}\cdot\text{mL}^{-1}$  to the saturation of response at  $150 \mu\text{g}\cdot\text{mL}^{-1}$ . However, after an elicitation producing a burst, subsequent elicitation by oligoguluronates were ineffective for at least 3h, showing a desensitizing effect of the elicitors on kelp responses (Küpper et al., 2001). Interestingly, these responses to alginate oligosaccharide only occur in sporophytes of *L. digitata*, whereas gametophytes show no response to these elicitors. For sporophytes, younger algal parts were more sensitive to elicitation than meristematic and older blade tissues. Subcellular localization of ROS investigated by confocal microscopy was found around epidermal and outer cortical cells, but not in the medulla which does not show sensitivity to elicitation (Küpper et al., 2001). Moreover, it was found that recognition and response to oligoguluronate with induction of an oxidative burst are restricted to the Laminariales (such as *Laminaria digitata*, *L. hyperborea*, *L. ochroleuca*, *Macrocystis pyrifera*, *Lessonia nigrescens*, and *Saccorhiza polyschides*), while Fucales constitutively release high quantities of hydrogen peroxide and Ectocarpales generally do not respond or weakly to elicitor addition (Küpper et al., 2002). The roles of ROS emitted during the oxidative burst were investigated on *L. digitata* pathogens and epiphytes, and it appears that  $H_2O_2$  inhibited their growth and survival at low concentrations (Küpper et al., 2001). Thus, accumulation of ROS following elicitation by oligoguluronate is essential in kelps for defense system and resistance set up against pathogens.

Induction of an oxidative burst in *L. digitata* was also demonstrated upon treatment with exogenous elicitors, lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria (Küpper et al., 2006). LPS were already described as inducers of defense mechanisms such as oxidative burst in metazoans (Remer et al., 2003) and in land plants (Meyer et al., 2001). The treatment of *L. digitata* thalli

with LPS from *Salmonella abortus equi* was shown to induce a strong release of hydrogen peroxide into the surrounding medium, in a similar amount range compared to elicitation by oligogulonates (Küpper et al., 2006). LPS and lipoteichoic acids (LTA; from the cell envelopes of gram-positive bacteria) both modify patterns of protein expression in the red alga *Chondrus crispus*, strongly suggesting that they are also perceived by this alga (Weinberger, 2007). Thus, LPS can also be considered as exogenous elicitors for marine algae.

The oxidative burst is directly linked to the activation of the plasma membrane-associated flavoenzyme NADPH oxidase, which seems to be a conserved component of the ancestral immunity system and thus an ancestral trait of innate immunity in eukaryotes (Weinberger, 2007). Indeed, the main subunits of NADPH oxidase in mammalian phagocytes, vascular plants, diatoms, and the red alga *Chondrus crispus* are structurally related (Keller et al., 1998; Torres and Dangl, 2005; Hervé et al., 2006). Studies of the oxidative burst response in *Gracilaria sp.* revealed that it was highly sensitive to diphenylene-iodonium (DPI), a specific inhibitor of NADPH-dependent enzymes, and to other inhibitors of flavoenzymes. NADPH oxidase was thus supposed to be the source of ROS after elicitation of *Gracilaria sp.* (Weinberger et al., 2005), as already observed in vascular plants and metazoans (Torres and Dangl, 2005).

### 3. Algal defense and biosynthesis of oxidized fatty acid derivatives

Perception of external molecular signals triggering production of fatty acids and their oxidized derivatives, oxylipins, was shown to be another common feature in land plants, animals as well as in macroalgae (Weinberger, 2007). In plants, the enzymatic processes involved in generation of oxidized fatty acids derivatives and other secondary metabolites, known as oxylipin pathway, proceeds from catalytic oxygenation of free polyunsaturated fatty acids (PUFAs) either by autoxidation or by intervention of lipoxygenases (LOXs), generating hydroperoxides, until production of aldehydes and alcohols by enzymes like allene oxide synthases (AOSs), hydroperoxide lyases (HPLs), and divinyl ether synthases (DESS) (Feussner and Wasternack, 2002). Red and brown macroalgae produce oxylipins from C18 and C20 fatty acids, which could be involved in algal defense (Potin et al., 2002; Pohnert, 2004). After exposure to cell-free extracts of the endophyte *Acrochaete operculata*, two lipoxygenases were upregulated in the red alga *Chondrus crispus*, one day after elicitation (Bouarab et al., 2004), displaying a crucial role of oxylipin signaling in resistance of *C. crispus*. Similarly, treatment of *L. digitata* thalli with LPS from *Salmonella abortus equi* was shown to induce free fatty acids and oxylipins (Küpper et al., 2006). Red algae, such as *Chondrus crispus*, feature both C18- and C20- derived oxylipins, which could also play a role as secondary defense signals in this eukaryotic lineage. For instance, treatment of *C. crispus* by methyl jasmonate activated the metabolism of C20 and C18 polyunsaturated fatty acids, inducing an elevated production hydroperoxides and cyclopentenones such as prostaglandins and jasmonates, a transient resistance to *A. operculata*, as well as an increase of stress

gene transcription (Bouarab et al., 2004). Moreover, jasmonic acid was described in vascular plants after derivatization of C18 and C20 oxylipins. In an evolutionary point of view, it is likely that jasmonate signaling have evolved between the primary and the secondary endosymbiotic events and is therefore considered as a putative common trait of photosynthetically eukaryotes, plants, micro- and macroalgae (Bouarab et al., 2004).

Most of recent studies on oxylipin pathways in Stramenopiles originate from investigations on diatoms. In the diatoms *T. rotula* and *S. costatum*, the sources of the fatty acid derivatives are lipids coming from galactolipids of the chloroplast membranes and from phospholipids of the plasma membrane (Fig 7a; Fontana et al., 2007). The fact that unicellular diatoms mobilize these existing resources presents advantages in rapid reactions against herbivores, when transcription and *de novo* biosynthesis of enzymes would be unlikely in wound-activated defense (Pohnert, 2005).

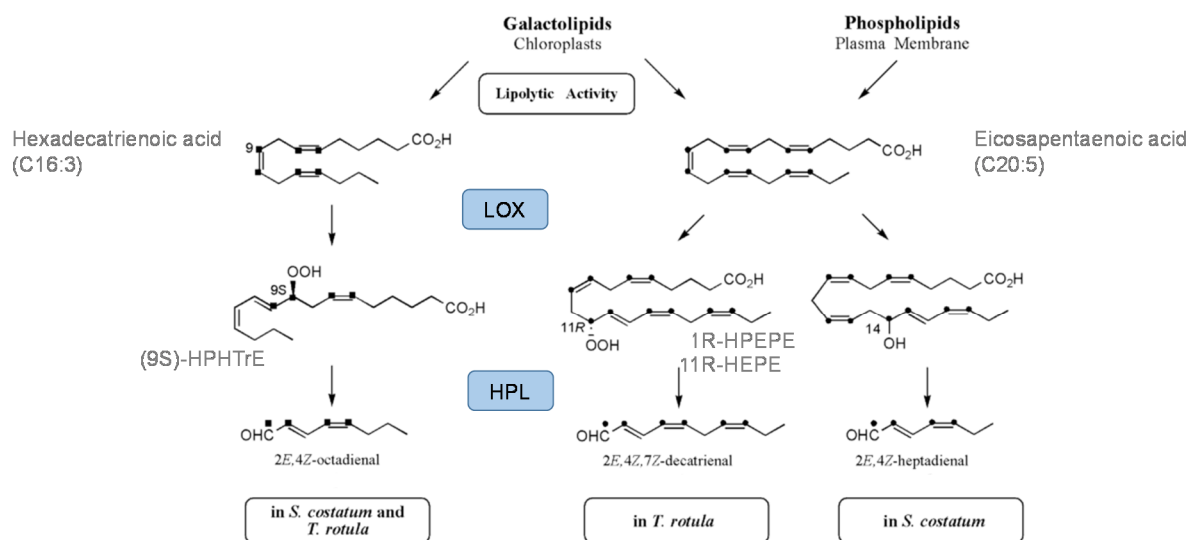
Wounded or mechanically damaged diatoms contain phospholipases A2 which activate and trigger release of free polyunsaturated fatty acids (PUFAs) (Pohnert, 2004). Nevertheless, it has been shown that healthy diatoms do neither contain free mono- nor polyunsaturated fatty acids, but only saturated fatty acids such as C14:0 or C18:0 (Pohnert, 2002). Only wounding of algae can induce a rapid production of fatty acid derivatives as well as a strong increase of free fatty acid amounts, such as eicosapentaenoic acid (C20), C18 and C16 fatty acids, few seconds after cell disruption (Pohnert, 2002; Pohnert, 2005). This leads to high local concentrations of these defensive compounds or of related potentially active aldehydes in the vicinity of diatom cells (Pohnert, 2000). It has been proposed that, following wounding, fatty acids were oxidized either chemically by autoxidation or enzymatically (Blée, 1998). Later on, studies showed that the diatoms *T. rotula* and *S. costatum* possess predominantly enzymatic systems for PUFAs' transformation. They are first oxidized via a lipoxygenase (LOX), producing hydroperoxides, and then a hydroperoxide lyase (HPL) which generates aldehydes (Pohnert, 2004). Generation of fatty acid derivatives by lipoxygenase's activity depends strongly on free polyunsaturated fatty acid availability in cells (Pohnert, 2002; Pohnert, 2005) and works independently from the Phospholipase A2 activity (Pohnert, 2002). Given that healthy diatoms only contain saturated fatty acids and that these fatty acids cannot be transformed by LOX (Pohnert, 2005), intact diatoms do neither contain nor release aldehydes (Pohnert, 2000). In the diatom *S. costatum*, generation of the aldehyde octadienal in wounded cells by oxidation of the fatty acid hexadecatrienoic acid (C16:3 w-4) was shown to involve LOX/HPL activity, thus not spontaneously synthesized by autoxidative process (D'Ippolito et al., 2003). The hydroperoxide intermediate was then described in *T. rotula* as (9S)-hydroperoxyhexadecatrienoic acid ((9S)-HPHTrE) formed by a 9S lipoxygenase (D'Ippolito et al., 2006). Moreover, Pohnert et al. (2002) described the synthesis of decatrienal as originating exclusively from eicosapentaenoic acid (EPA, C20:5 w-3) probably by an 11R-lipoxygenase, via the hydroperoxide 11R-HEPE (Pohnert, 2000), and decadienal coming from arachidonic acid (C20:4). In contrast, the author did not find any of these aldehydes originating from C18 and C22 fatty acids. Thus, *T. rotula*

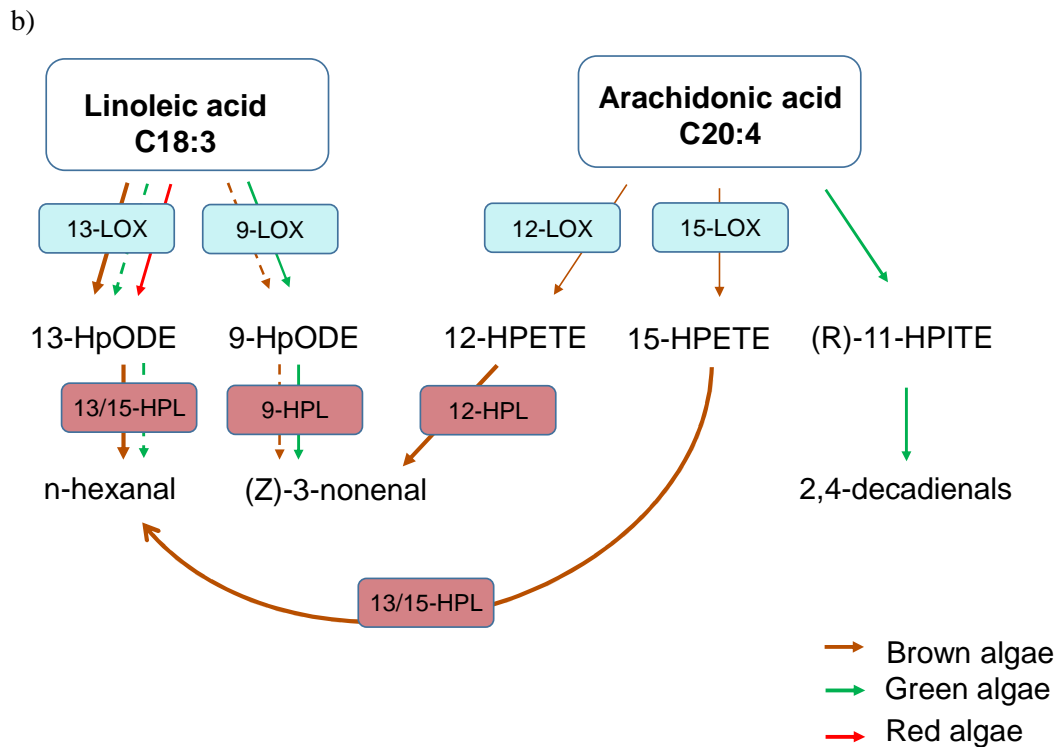
shows a substrate specificity for C20 fatty acids for production of oxylipins and aldehydes. In the same study, it appears that free fatty acids are required as lipoxygenase substrates, as conjugated C20 fatty acids did not induce subsequent production of aldehydes. Moreover, it has been shown that the generation of decatrienal is a rapid reaction, as *T. rotula* cells produce 4.1 fmol of this aldehyde per cell within 1 – 3 min after cell damage (Pohnert, 2000).

In macroalgae, fatty acid derivatives originating from the oxylipin pathway were shown to have attracting roles as sexual pheromones in brown algae (Pohnert and Boland, 2002), as well as defensive roles against epiphytes (Lion et al., 2006), endophytes (Bouarab et al., 2004) and herbivores (Rempt et al., 2012). As previous investigations on diatoms showed major repellent effects of oxylipins on copepods, studies on their ecological effects and their characterization began in macroalgae. Among green algae, studies on *Ulva sp.* composing green tides showed that sea-lettuce like but not tube-like morphotypes produced high amounts of volatile aldehydes upon tissue damage (Alsufyani et al., 2014). 2,4-heptadienal and 2,4-decadienal were the most abundant aldehydes produced by damaged *Ulva sp.*, whereas 2,4,7-decatrienal was produced in less quantity. These aldehydes are derived from omega-3 and omega-6 PUFAs mostly with 18 carbon atoms such as stearidonic acid (C18:4 n-3), alpha-linolenic acid (C18:3 n-6), linoleic acid (C18:2 n-6), and monounsaturated oleic acid (C18:1 n-9) and palmitoleic acid (C16:1 n-7) (Fig 7b). Moreover, they were marginally derived from C20 fatty acids like EPA (C20:5 n-3) and arachidonic acid (C20:4 n-6). Transformation of these PUFAs is performed with LOX, 11-LOX acting on eicosanoids and 9-LOX on octadecanoids (Alsufyani et al., 2014), forming hydroperoxides and finally PUAs. In the red algae *G. vermiculophylla* and *G. chilensis*, Rempt et al. (2012) found that the oxylipin 7,8-di-HETE active against *E. peruviana* was derived from arachidonic acid, through a lipoxygenase-mediated transformation. The release of arachidonic acid and the following hydroxylated compounds come from a phospholipase A, probably directly from galactolipids (Lion et al., 2006). In brown algae, investigations of fatty acids and derivatives and their biosynthetic pathways were already described in *Laminaria angustata* (Boonprab et al., 2003a; Boonprab et al., 2003b). This brown alga forms C6 and C9 aldehydes enzymatically, from two different pathways. C6 aldehydes are derived either from C18 or from C20 fatty acid and C9 aldehydes are formed exclusively from the C20 fatty acid arachidonic acid. The C18 fatty acid linoleic acid is converted to the hydroperoxide 13-hydroperoxyoctadecadienoic acid (13-HPODE), then cleaved to form the aldehyde n-hexanal. Interestingly, the C6-aldehyde-forming branch in the brown alga had almost the same properties as that in higher plants. On the contrary, the C9-aldehyde forming branch uses preferentially arachidonic acid instead of linoleic acid as substrate (Boonprab et al., 2003a). Moreover, it seems that the HPL which catalyzes the formation of C9 aldehydes is highly specific to hydroperoxides from C20 fatty acids (Boonprab et al., 2003b). In the kelp *L. digitata*, Küpper et al. (2006) found that free fatty acids were produced upon application of LPS from *Salmonella abortus equi* and *Marinobacter hydrocarbonoclasticus*. Hence, linoleic acid (C18:2), linolenic acid (C18:3), arachidonic acid (C20:4),

and eicosapentaenoic acid (C20:5) were produced highest amounts after 60 min following the LPS treatment. Other fatty acids such as myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1) were also detectable upon 30 min of LPS treatment. Moreover, oxylipins were found in the kelp after LPS treatment, like 13-hydroxyoctadecatrienoic acid (13-HOTrE) and 15-hydroxyeicosapentaenoic acid (15-HEPE). Investigations on the effect of the endogenous elicitors oligogulonates were first reported to fail at inducing significant amounts of free fatty acids and oxylipins in *L. digitata* (Küpper et al., 2006). However, Goulitquer et al. (2009) demonstrated that a wide range of aldehydes such as hexenal, hexanal, 4-hydroxyhexenal, nonenal, 4-hydroxynonenal or dodecadienal were emitted by *L. digitata* upon elicitation by oligogulonate. In addition, it appears that the aldehyde 4-HHE is able to induce significant production of the oxylipin 13-hydroxyoctadecatrienoic acid (13-HOTrE) after 24 hours. Similar results were obtained in this kelp in response to copper stress, which induced production of a wide variety of fatty acids, oxylipins as well as amino acids (Ritter et al., 2014).

a)





**Figure 7.** Representation of the oxylipin pathway in diatoms (a; from Fontana et al., 2007) and in brown, green and red algae (b).

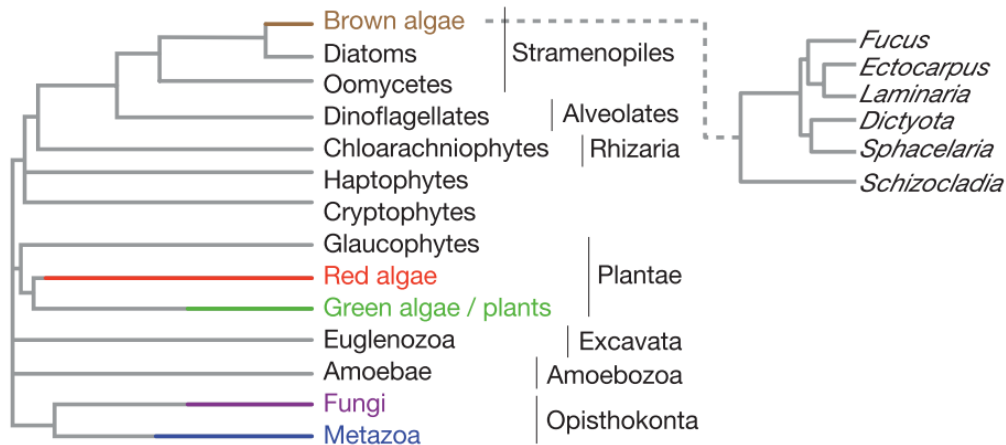
## IV. Brown algal signaling and defenses against herbivores

### 1. Interests of studying kelps

#### a. Evolutionary aspects

Brown algae belong to the Phaeophyceae and constitute complex photosynthetic organisms with a very different evolutionary history from land plants and other multicellular Eukaryotes (Fig 8; Cock et al., 2010). Like red and green algae, land plants, Fungi and Metazoa, brown algae have independently evolved complex multicellularity. Moreover, brown algae share the same evolutionary history as the diatom lineage, and can also feature ancestral traits that are common in other eukaryotic lineages as well as convergent traits that appeared several times independently. As described above, some aspects of defense mechanisms in brown algae can be conserved such as the perception of MIMPs or the oxidative burst response, while others may be specific to the Stramenopiles lineage.



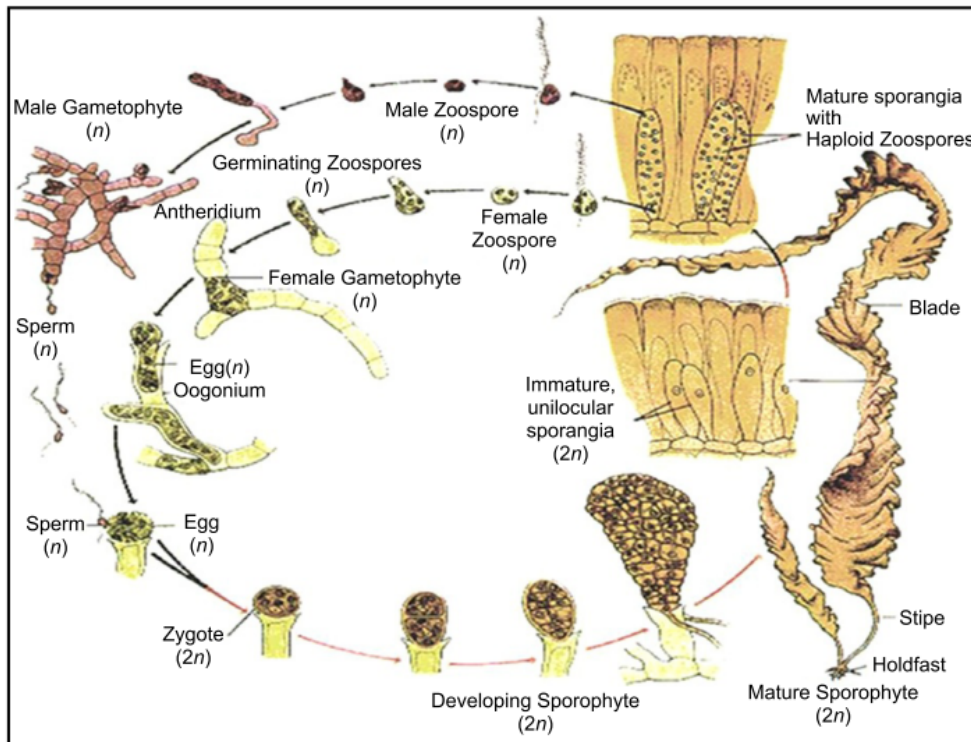


**Figure 8.** Simplified representation of the evolutionary tree of the eukaryotes showing the five major groups that have evolved complex multicellularity (indicated in color). Kelps are represented by the genus *Laminaria*. From Cock et al. (2010)

## b. Ecological characteristics

The Laminariales order, also known as kelps, constitute ecologically important macroalgae in coastal ecosystems on Earth. Kelps form sub-marine forests dominating coastal rocky shores of cold to temperate zones around the world (Steneck et al., 2002). They are the most important biogenic structures in benthic marine ecosystems, constituting a true habitat providing protection and food source for a wide variety of other organisms such as other macroalgae, molluscs, crustaceans, fish and mammals (Bartsch et al., 2008; Smale et al., 2013). Kelp forests are thus one of the most diverse and productive ecosystem on Earth and can be compared to terrestrial tropical forests. Among the ten families forming the Laminariales, the families Laminariaceae, Lessoniaceae and Alariaceae constitute the most common species in these kelp forests.

Kelp life cycle is characterized by an alternation of generations with heteromorphic free-living life stages (Fig 9). One phase is defined by a large diploid sporophyte and the other is constituted by a microscopic haploid gametophyte generation where males and females differentiate and mate to restore diploidy (Kim and Bhatnagar, 2011). On the distal parts of the kelp, sori which are regions of epidermis along the length of the blade, contain haploid meiospores. Once released in the medium, these meiospores settle on the substrate, germinate and produce a microscopic filamentous gametophyte. The male gametophytes produce the motile, biflagellate sperm, while the female gametophytes transforms the distal part of its filament into an elongated oogonia containing one egg. After fertilization, the zygote germinates to form a flat embryo that subsequently differentiates into the mature sporophyte (Fig 9).



**Figure 9.** Kelp life cycle showing an alternation of generations with heteromorphic individuals. From Kim and Bhatnagar (2011).

c. Economic interests of kelps

Mainly collected for alginate extraction used in food, pharmaceutical and textile industries for its gellant, emulsifier or texturing properties, kelps provide huge economic interests (Bartsch et al., 2008; Kim and Bhatnagar, 2011). In medicine, *Laminaria* species have important interests for their content in diverse polysaccharides, for the production of wound dressing for the skin or the stomach, and encapsulation of medicines. Kelps are also intensively harvested for abalone feeding, and some specific sources of human diet (e.g. Kombu and Wakame). These seaweeds are also under study for development of aquaculture, as harvesting kelps in their natural environment induces important modifications of ecosystems which imposes to manage finely the algal resources in natural populations (Bartsch et al., 2008).

## 2. The thesis project

a. Scientific context and problematic of the thesis

In the kelp *Laminaria digitata*, defense elicitation with alginate oligosaccharides triggers a rapid oxidative burst and concomitant release of VOCs, as well as a transcriptional reprogramming before resistance establishment (Küpper et al., 2001; Küpper et al., 2002; Cosse et al., 2009; Goulitquer et al., 2009). In laboratory-controlled experiments, the effects of specialist herbivores have also been shown

to specifically regulate gene transcription in this alga (Leblanc et al., 2011). Moreover, chemical cues present in the kelp bed and/or released from elicited brown algae induced modifications of the physiology of neighboring *L. digitata*, in response to defense elicitors (Thomas et al., 2011). These results suggested the occurrence of water-borne signaling and revealed some physiological responses, similar to the priming responses in land plants. More recently, a systemic reaction was highlighted in *L. digitata* following elicitation on a distant area of the frond. These distant responses included an oxidative burst, an increase in haloperoxidase activities and a stronger resistance against herbivory (Thomas et al., 2014). Pharmacological inhibitors further suggested that the liberation of free fatty acids could play a key role in systemic signaling, reminiscent of what is known in land plants.

In the independent brown algal lineage, we could question the existence of universal mechanisms of defense and distance communication that have evolved in kelps during biotic interactions with herbivores. In the marine environment, most of primary consumers are generalist, but in kelps, few specialist interactions with herbivores have been described. We were especially interested in better understanding the defense responses and signaling processes in two kelp species involved in specialized interactions with their herbivores. The first interaction occurs between *Laminaria digitata* and its specialist herbivore *Patella pellucida* (Fig 10a) on the coastal rocky shore in Brittany. *Laminaria digitata* (Hudson) Lamouroux 1813 has been widely studied in its natural environment, revealing a high trophic specialization of this kelp by only one species, the gastropod *Patella pellucida* (formerly *Helcion pellucidum*), even if other species can also feed on this kelp but only temporally (Schaal et al., 2010; Leblanc et al., 2011). The second specialized interaction implies the kelp *Lessonia spicata* with its specialist and selective herbivore *Scurria scurra* Lesson 1830 (Fig 10b; Meynard, 2014). *L. spicata* (Suhr) Santelices (previously *L. nigrescens*) is one of the most dominant habitat-forming macroalgae in the low intertidal zone in central and southern Chile, producing high amounts of biomass (Santelices et al., 1980; González et al., 2012). The stipes of *L. spicata* are the habitats of the limpet *S. scurra*, digging holes on the stipes and holdfasts of this kelp, where it seems to live and feed, thereby structurally weakening the host (Santelices et al., 1980)

a)



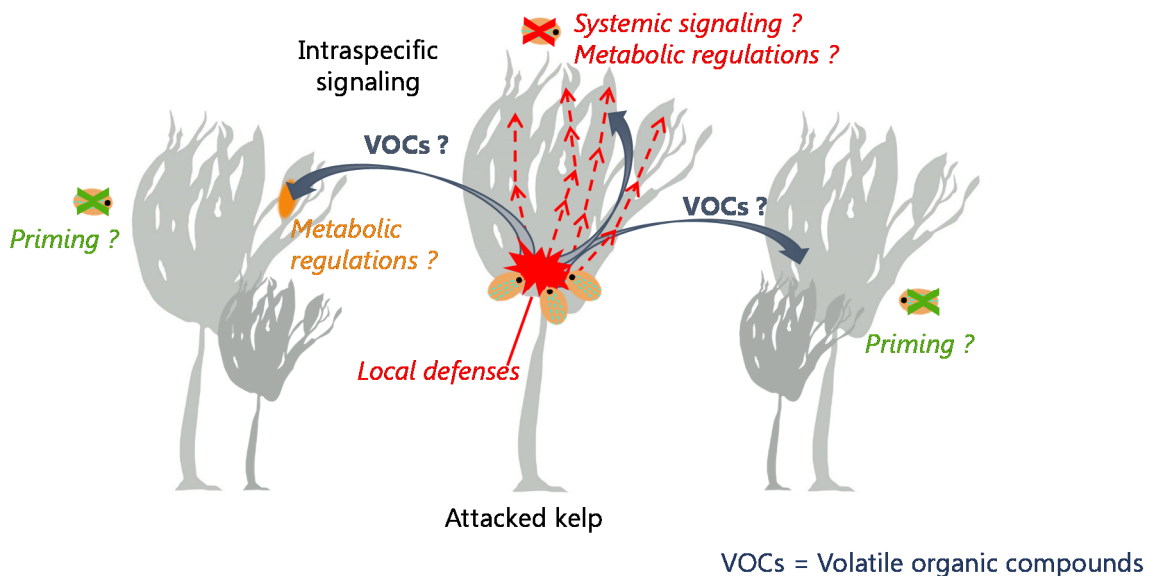
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b)



**Figure 10.** Pictures of *Laminaria digitata* and its specialist herbivore *Patella pellucida* in Brittany (a) and of *Lessonia spicata* and its specialist herbivore *Scurria scurra* in Chile (b).

In the context of defense responses and distant chemical signaling in brown algal kelps during interactions with specialist herbivores (Fig 11), the thesis aims at addressing the following questions: 1) what are the local algal responses following grazing by specialist herbivores? 2) what are the waterborne cues involved in distance signaling? 3) what are the effects of these signals on kelp defenses against herbivores, and are these responses dependent on the degree of specialization of the herbivore with the alga? 4) which modifications does this signaling trigger on metabolic regulations of the receiving kelp?



**Figure 11.** Schematic representation of potential chemical communication in kelps leading to priming. Upon grazing attack, a kelp responds locally directly against herbivores by deterrent or toxic compounds. Grazing can also induce a systemic signaling in the whole kelp, leading to defense in distal algal parts. Finally, a chemical distance signaling could be triggered via Volatile Organic Compounds (VOCs), potentially inducing metabolic regulations in the signal-receiving kelp and resulting in priming.



## b. Thesis project and outline

In order to address the above questions in relationship with the chemical ecology research domain, combined approaches of chemistry and ecology were developed. Chemical analyses were performed using metabolomic approaches on algal exo- and endo-metabolome by LC-MS and GC-MS methods, while some ecological questions were investigated through deterrence and preference bio-assays with specialist or generalist herbivores. Bio-assays were preferentially performed in *L. spicata* as experimental conditions in Las Cruces facilitated the set-up of this type of systems, providing current seawater and air flows, while physiological and metabolomic analyses were preferably carried out on *L. digitata* because of the availability of the measuring instruments and chemical reagents in Roscoff.

This manuscript is divided in three chapters followed by conclusions and future prospects. In the first chapter, local mechanisms and potential signaling of defense against grazers are studied in both kelp species *L. digitata* and *L. spicata*. A first part of this chapter is dedicated to the article about herbivore-induced chemical and molecular responses of the kelps *Laminaria digitata* and *Lessonia spicata*. To complete this chapter, results on emitted compounds in the surrounding seawater of *L. digitata* after challenging by elicitors are presented.

The second chapter is devoted to the effects of distance signaling on the interaction between the kelp and herbivores. This chapter starts with the article dealing with ecological and chemical changes in *L. spicata* to grazing by specialist herbivores. In this article, algal deterrence is tested by non-choice experiments after direct or indirect induction of stress, by measuring algal consumption by herbivores. Complementary results then provide precisions regarding preference towards direct or indirect stress induction of algae. Choice tests were performed in the laboratory on *L. digitata* with the generalist herbivore *Haliotis tuberculata*, and on *L. spicata* with both its specialist herbivore *S. scurra* and a generalist herbivore *Tegula atra*. A preliminary experiment was additionally carried out *in situ* to test the attraction of *L. spicata* for different natural or simulated grazing treatments.

The third chapter focuses on studies of aldehyde-based chemical signaling and its effects on algal metabolome and on the interaction between kelps and herbivores. The article related to this chapter reports the algal physiological and metabolic regulations after application of aldehydes on *L. digitata*, as well as their deterrent effect on the grazer *P. pellucida*. Additional results deal with the effects of incubation of *L. spicata* in aldehydes against the generalist herbivore *T. atra*, by measurement of algal consumption by grazers.

Finally, integration of all these results allows to draw a picture of the chemical communication in kelps in response to grazing stress, especially via signaling by aldehydes. This thesis opens new research lines that are discussed in the final part of the manuscript.

# **Materials and methods**

## I. Biological material

### 1. *Laminaria digitata* / *Patella pellucida*

Experiments on *L. digitata* were conducted alternatively with both wild sporophytes (i.e. born in natural populations) and individuals grown in the laboratory from spores collected in the field. Wild *L. digitata* were collected at Roscoff (48°43'31"N ; 3°58'8"O) at low tide at coefficient 90 or higher. The whole young plantlet of 20 to 30 cm long was delicately taken for the rocks and brought back to the lab within 1h. There, algae were stored in tanks with 0.45 µm filtered seawater and constant air supply, at 13±1°C. Light was provided by Philips daylight tubes at a photon flux density of 40 µmol.m<sup>-2</sup>.s<sup>-1</sup> for 12h per day. Seawater was changed every 3 – 4 days.

To reduce inter-individual variability in kelp responses to biotic stresses, laboratory-born individuals were produced from mature adult wild sporophytes of *L. digitata* collected in the same populations. They were obtained as unialgal cultures grown from random crosses of gametophytes recovered in the laboratory. Developing sporophytes were then transferred in larger flasks in sterilized seawater together with a Provasoli Enriched Seawater (PES) culture media (10 mL of Provasoli per liter of water). They were placed at 13±1°C with constant bubbling in the same light conditions as wild kelps and grown until they reached a size of about 3 to 4 cm. The medium was changed every week. When algae were ~2.5 cm long, PES supply was stopped and filtered seawater was put instead of sterilized seawater.

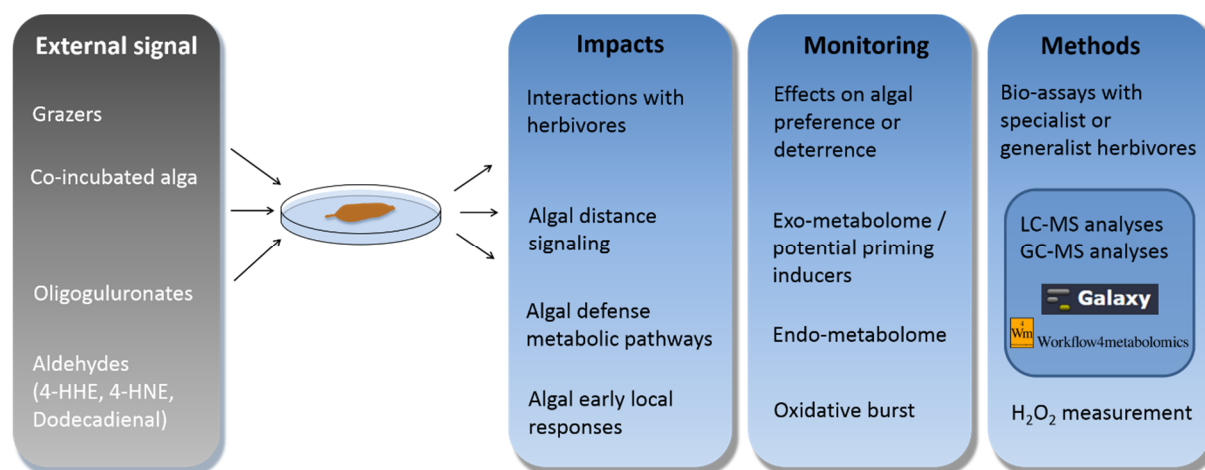
*Patella pellucida* were collected at low tide on the same site as *L. digitata*, in Roscoff. Animals from 1 to 5 mm were softly removed from the meristem or the stipes of *L. digitata*. They were acclimated in an aquarium with constant flow of seawater at 16±1°C and air blowing, and fed with *L. digitata* sporophytes tissue while awaiting experiments.

### 2. *Lessonia spicata* / *Scurria scurra* and *Tegula atra*

*Lessonia spicata* plantlets and their herbivores *Scurria scurra* and *Tegula atra* were collected at Las Cruces in Chile (33°30'09"S : 71°37'59"O), aside from the marine reserve belonging to ECIM (Estación Costera de Investigaciones Marinas) marine station. Juvenile kelps were harvested entirely, by removing the holdfast from the rocks. Individuals consisted in a holdfast with all the stipes emerging from it, with a total length between 20 and 50 cm. Plantlets were brought to the laboratory within an hour and put together in a tank at ambient temperature, with constant seawater flow and blowing air, without direct sunlight, for acclimation to the lab. *S. scurra* and *T. atra* grazers, between 13 and 28 mm in diameter, were collected in the same place, softly removed from the stipes of *L. spicata*. In the lab, they were fasted for 48h under constant seawater flow before using for experiments.

## II. Algal laboratory treatments

The whole thesis is based on a general methodology presented in Fig. 12 and the different treatments and experiments applied to each kelp species are detailed in Table 1. The distribution of experiments in *L. digitata* and *L. spicata* shows a clear complementarity between chemical analyses and bio-assays carried out in each model species, based on different expertise and facilities available in ECIM and Roscoff's laboratories.



**Figure 12.** General methodology used in the whole thesis project. After application of biological or chemical external signals, impacts on local to distant levels were investigated via methods of bio-assays, metabolomic analyses and hydrogen peroxide measurement.

**Table 1.** Details of treatments and experiments applied to each kelp species *L. digitata* (X) and *L. spicata* (X).

	Algal local early responses	Algal distance signaling and defense metabolic pathways		Interactions with herbivores	
	Oxidative Burst	Exo- and endometabolome		Effects on algal preference and deterrence	
	H <sub>2</sub> O <sub>2</sub> quantity measurement	Metabolomic analyses		Bio-assays : choice and non-choice tests	
Grazing			X		X
Co-incubation	X	X	X		X
Elicitation by GG	X	X		X	X
Aldehydes	X	X		X	X



In order to evaluate impacts of biological and chemical external signals on algal responses in terms of early local responses, in terms of metabolism modifications and interactions with herbivores, different treatments were tested on both kelp species *L. digitata* and *L. spicata*.

## 1. Control

The kelp was put in seawater, without any treatment, in the same conditions as the compared treated kelps.

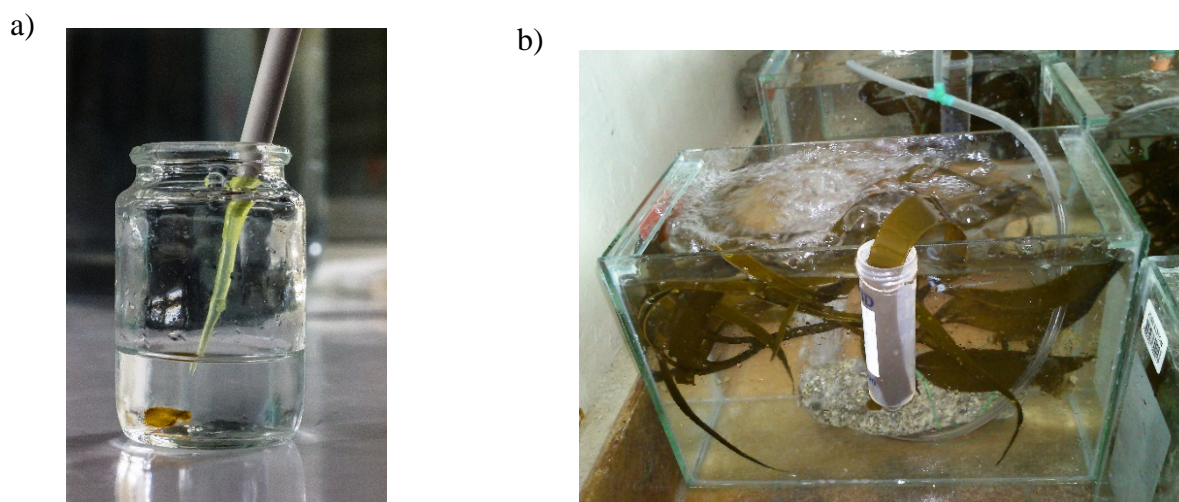
## 2. Grazing by herbivores

To determine the effects of a natural grazing on kelp defense and chemical communications, including other effects than mechanical wounding on algal responses such as effects of herbivore saliva, exposure of plantlets to grazers was performed in each kelp-herbivore pair. Herbivores were first starved during 24h to 30h before grazing and they were then placed directly on the frond for 24 to 48h, according to the experiment. This grazing treatment was exclusively performed with *L. spicata*'s grazers *S. scurra* and *T. atra*.

## 3. Elicitation by oligoguluronate

Using oligoalginate as endogenous elicitors constitutes a simple and standardized way to mimic an herbivore attack, assuming that herbivore consumption is accompanied by a partial digestion of cell wall polysaccharides that releases oligoalginate in the environment. The standardized quantity of elicitor used in these experiments avoids the inter-individual variability of herbivore appetite or consumption rate, influencing stress applied to the kelp. Elicitation was performed with poly-alpha-1,4-L-guluronic acid blocks (GG blocks) prepared by acid hydrolysis of sodium alginate from *L. digitata* stipes (Danisco, Landerneau, France), following Haug et al. (1974). A solution of 0.5% alginates was hydrolyzed at 100°C for ~4h with 0.3 M hydrochloric acid. The three types of oligosaccharides obtained, oligoguluronates (GG), oligomannuronates (MM) and hybrid oligosaccharides (MG), were separated by alternating centrifugation and selective precipitation steps. The oligoguluronate (GG) fraction was dialyzed, lyophilized and analyzed by gel filtration for testing for purity.

The homopolymeric GG blocks were used as an elicitor at a final concentration of 150 µg.mL<sup>-1</sup>, as described in Küpper et al. (2001). Treatments were performed differently according to the kelp species. Indeed, *L. digitata* plantlets, from 2.5 to 4 cm long, were elicited in 30 mL of filtered seawater, allowing a treatment of the whole thallus (Fig 13a). For *L. spicata*, individuals were bigger and only one isolated frond (10 to 15 cm long) per individual was treated in 50 mL Falcon tubes filled with GG solution (Fig 13b) and later refereed as elicited alga. If the total amount of elicited tissue was different between the two species, the external elicitation was applied in similar conditions.



**Figure 13.** Pictures of elicitation set up in *L. digitata* (a) and in *L. spicata* (b). Laboratory-grown *L. digitata* were small enough to be entirely immersed in GG solution, while fronds of *L. spicata* were partly incubated in a Falcon tube containing the GG solution at  $150 \mu\text{g.mL}^{-1}$ .

#### 4. Co-incubation of two kelps

Determination of waterborne signals effects on algal defense from a neighboring kelp naturally involves a contact of seawater between the two kelps. Thus, and in order to stay close to conditions in nature, co-incubation of two kelps was performed, allowing chemical emission and potentially simultaneous perception by the neighboring kelp.

#### 5. Incubation in aldehyde solutions

As aldehydes were shown to be emitted after GG elicitation of *L. digitata* (Goulitquer et al., 2009), it is supposed that they could play a role in chemical communication between kelps, then perhaps having an effect on their interactions with grazers. Thus, some aldehydes from Thomas et al. (2011) were chosen and applied to *L. digitata* or *L. spicata* fronds at different concentrations. We chose the 4-hydroxy-2-hexenal (4-HHE;  $10 \text{ mg.mL}^{-1}$ ), 4-hydroxy-2-nonenal (4-HNE;  $10 \text{ mg.mL}^{-1}$ ) both obtained from Cayman Chemical, and 2,4-Dodecadienal ( $20 \text{ mg.mL}^{-1}$ ) from Sigma-Aldrich. For application in experiments, they were diluted in EtOH 70%.

### III. Experimental setups

#### 1. Exo-metabolome of an elicited alga

Wild *L. digitata* were either elicited with GG solution at  $150 \mu\text{g.mL}^{-1}$  or let as controls in filtered seawater for 30 min at ambient temperature. Four replicates were made, each containing 4 algae put

together in 100 mL filtered seawater. Algae were rinsed three times for 5 min with filtered seawater and placed individually in 50 mL filtered seawater. After 1h, 4h, 12h and 24h, the surrounding seawater was recovered, and algae were blotted dry and weighted. Both algae and seawater were frozen in liquid nitrogen and conserved at -80°C until metabolomic analyses.

## 2. Co-incubation of *L. spicata*

### a. Development of general laboratory experimental set up

The experimental unit consisted in two *L. spicata* plantlets placed in a 40 liters aquarium with constant seawater and air supplies. One of them was submitted to a stressful treatment, either chemical elicitation by oligoguluronates or direct grazing by *S. scurra*, and the other kelp was co-incubated with the treated one. In parallel, two control plantlets were manipulated in the same way, but without any elicitor or grazer. The co-incubation of the treated individual with its neighbor (or the respective controls) lasted 24 or 48 hours, according to the type of treatment (see below for details). Independently, the autogenic changes of algal mass was assessed during the whole period of the experiment and checked for the different treatments of the *L. spicata* plantlets. For this, a separate set of experimental units with the same elicitation treatments was dedicated to estimate biomass changes for the duration of the grazing experiment, but kept without any further grazing.

### b. Grazing experiment

Two individuals of *S. scurra* were first deposited on one frond of 10 *L. spicata* plantlets kept in a net, to avoid herbivore escape. The kelp in the net was named DG (direct grazed), whereas the other was named IG (indirect grazed, e.g. not directly in contact with the grazer, but co-incubated with the DG alga). The controls used for each treatment were named C<sub>DG</sub> (Control direct grazed) and C<sub>IG</sub> (Control indirect grazed) respectively. After 48 hours of co-incubation, the most grazed frond of DG and one frond of IG, C<sub>DG</sub> and C<sub>IG</sub> plantlets were selected for the bio-assay. The Grazing experiment was independently repeated three times.

### c. Elicitation experiment

Chemical elicitation was done by placing, during 1 hour, one frond of the directly elicited individual (E) in a 50 mL-Falcon tube filled with 150 µg.mL<sup>-1</sup> GG dissolved in seawater. The Falcon tube was adjacent to the aquarium so the integrity of the individual was kept, and no more than 5 cm of frond tissue was exposed to air, between the aquarium and the tube. The control treatment was identical, except for the Falcon tube that was filled with seawater only. The separately-treated fronds (E and C<sub>E</sub>) were then rinsed with seawater and restored in the aquarium, with the co-incubated *L. spicata*. In August, seawater was renewed one hour after elicitation and let in the aquarium for 24 hours. In November,

plantlets were placed in aquarium with constant water supply. Fronds elicited in GG solution (E ; Elicited) or immersed in seawater (C<sub>E</sub> ; Control elicited), and fronds from the co-incubated kelps (IE and C<sub>IE</sub> respectively for Indirectly Elicited and Control indirectly elicited) were cut at the stipe, blot-dried and weighted for the bio-assay. The Elicitation experiment was done three times.

### 3. Elicitation treatment of *L. digitata* for food preference test of *H. tuberculata*

Wild adult *L. digitata* from 60 cm to 1.20 m were treated either by elicitation with GG or as control and were then exposed to a feeding choice tests on abalones (*Haliotis tuberculata*). Treatments were done 3 days before the bio-assay. Elicitation was performed in 150 µg.mL<sup>-1</sup> GG for 1h with bubbling, while control algae were incubated in seawater. They were then placed separately in two tanks until the beginning of the choice test.

### 4. Grazing and co-incubation of *L. spicata* for food preference test of *T. atra*

*L. spicata* plantlets were exposed to different treatments, then presented as food choices for *T. atra* in Y tubes bio-assay. Algae were either treated as controls (without treatment), directly grazed by *T. atra* or by *S. scurra*, co-incubated with a kelp grazed by *T. atra* or by *S. scurra*. For grazing and co-incubation treatment with *T. atra*, one treatment (treatment <sup>(A)</sup>) was performed with grazing by 17 herbivores placed on 14 fronds of *L. spicata* for 36h and the co-incubation lasted 36h, and the other treatment (treatment <sup>(B)</sup>) involved 30 herbivores grazing on 12 fronds for 24h and the co-incubation lasted 24h. For *S. scurra* treatments, 17 herbivores were placed on 3 *L. spicata* fronds for 36h and the co-incubation lasted 36h. Grazed fronds were put individually in 6 L boxes with constant seawater flow, each upstream to an alga receiving the seawater flow from the grazed kelp and thus treated by co-incubation with a grazed kelp. Control fronds were put individually in boxes with seawater flow.

### 5. Attraction of natural or artificial grazing of *L. spicata* for *S. scurra in situ*

*In situ* experiments were carried out at Las Cruces on *L. spicata* individuals and their herbivores *S. scurra* in order to investigate the attraction of natural and artificial wounding on *S. scurra*'s behavior. Different treatments (Table 2) were applied to kelps and presence or absence of *S. scurra* on *L. spicata* individuals was noted after 1 and 5 days.

**Table 2.** *In situ* treatments of *L. spicata* tested on attraction of *S. scurra*.

<b>A</b>	<b>Attraction of an artificial wounding 24h after wounding generation</b>
	5 <i>L. spicata</i> with artificial wounding made at D0 1 <i>S. scurra</i> added on the wounding at D1
<b>B</b>	<b>Control artificial wounding</b>
	5 <i>L. spicata</i> with artificial wounding made at D0 No addition of <i>S. scurra</i>
<b>C</b>	<b>Control of grubbing-up effect</b>
	5 <i>L. spicata</i> with natural wounding and 1 <i>S. scurra</i> into the wounding Removing and putting back the herbivore in the wounding
<b>D</b>	<b>Attraction of the natural wounding</b>
	5 <i>L. spicata</i> with natural wounding and 1 <i>S. scurra</i> into the wounding Removing and putting the herbivore outside the wounding, on the same stipe
<b>E</b>	<b>Natural recolonization</b>
	5 <i>L. spicata</i> with natural wounding and 1 <i>S. scurra</i> into the wounding Removing and putting the herbivore on a neighboring stipe on the same <i>L. spicata</i> individual
<b>F</b>	<b>Observation of <i>S. scurra</i> in its wounding</b>
	5 <i>L. spicata</i> without treatment

## 6. Aldehyde incubation and elicitation treatments of *L. digitata*

Laboratory-grown *L. digitata* plantlets were submitted either to an elicitation, a control or an aldehyde treatment, at 13°C under agitation. They were incubated in filtered seawater and in GG solution at 150 µg.mL<sup>-1</sup> for 1h, for control and elicitation treatment, respectively. Aldehyde treatment lasted 4h and consisted in incubation of algae in 4-HHE, 4-HNE or in dodecadienal. 4-HHE was tested at 1 µg.mL<sup>-1</sup>, 500 ng.mL<sup>-1</sup>, 100 ng.mL<sup>-1</sup>, 50 ng.mL<sup>-1</sup> and 10 ng.mL<sup>-1</sup>; 4-HNE was tested at 1 µg.mL<sup>-1</sup> and 100 ng.mL<sup>-1</sup>; dodecadienal was only tested at 100 ng.mL<sup>-1</sup>. H<sub>2</sub>O<sub>2</sub> emission was measured before and during 1h incubation in all solutions. Then, algae were either used for metabolomic analyses in LC-MS, or in non-choice feeding bio-assays with *P. pellucida*.

## 7. Aldehyde treatment for *L. spicata* consumption studies by generalist grazers

*L. spicata* fronds were treated either with the 2 aldehydes 4-HHE and 4-HNE, both at 100 ng.mL<sup>-1</sup> and 10 ng.mL<sup>-1</sup> or as controls, or co-incubated with a grazed kelp by *T. atra*. Treatment by aldehydes lasted 4h, like for *L. digitata*, at room temperature. The co-incubation treatment was performed with a grazed kelp by 2 *T. atra*, placed upstream to a kelp receiving the seawater flow from the grazed kelp, thus treated by co-incubation with a grazed kelp. This co-incubation treatment lasted 24h at room temperature.

## IV. Hydrogen peroxide measurement by chemoluminescence

Kelps rapidly respond to grazing mimicry via application of exogenous or endogenous elicitors by emitting hydrogen peroxide (Küpper et al., 2001; Küpper et al., 2006), then providing higher algal resistance against pathogens (Küpper et al., 2002). Thus, we used the accumulation of H<sub>2</sub>O<sub>2</sub> contained in the surrounding seawater as an estimation of algal stress measurement. The concentration of hydrogen peroxide in the medium around algae was determined using the luminol chemiluminescence method (Glazener et al., 1991) with a Tristar luminometer (EG&G Berthold, Bad Wild-bach, Germany). This device is equipped with two injectors, bathed in two reagents: the luminol dissolved in DMSO solution (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) diluted at a final concentration of 0.3 M in distilled water and a horse-radish peroxidase (HRP; Sigma) in solution with 10 mM pH 7.8 phosphate buffer. The reaction between these two reagents and the hydrogen peroxide triggers emission of light, whose quantity is proportional to the concentration of H<sub>2</sub>O<sub>2</sub> in the sample:



An optimization of the luminometry measurement method was developed on the Tristar luminometer. For each measurement, 150  $\mu\text{L}$  of surrounding seawater were collected and put in 96-well microplates (Lumitrac 200, White, Greiner Bio-One). In the luminometer, after a first injection of 50  $\mu\text{L}$  of 20  $\text{U}\cdot\text{mL}^{-1}$  horse-radish peroxidase, the microplate was shaken for 1.0 s and 100  $\mu\text{L}$  of luminol solution were added to the sample. Then, chemoluminescence was recorded immediately after the last injection with a signal integration time of 0.55 s. Finally, after several tests of delays, a time delay of 10 s was introduced for device stabilization before the next measurement. This sequence was repeated for each well of the microplate. At the end, for calculating the concentration of H<sub>2</sub>O<sub>2</sub> present in the samples, calibration with a standard curve was carried out once during any series of measurements.

## V. Global metabolomic analyses

### 1. Metabolite extraction

Metabolome analyses were performed both on seawater surrounding algae (exo-metabolome) and on algal tissues (endo-metabolome). This allowed to investigate the potential waterborne signals emitted by kelps in the medium, together with the metabolites produced into the alga in response to a stimulus. Thus, different types of compounds were targeted: small compounds of molecular weight <1 kDa and bigger compounds. This implied different extraction methods and analysis techniques.

#### a. Solid-liquid extraction

Metabolite extraction was performed with 100 mg of frozen algal powder mixed with 1 mL MeOH:H<sub>2</sub>O (8:2), and placed under agitation during 1h at 4°C. In each sample, 1.25 µg.mL<sup>-1</sup> 12-OH-lauric acid was added as an internal standard for the negative ionization mode, and 10 µg.mL<sup>-1</sup> of reserpine for the positive mode. The MeOH phase was recovered and dried under N<sub>2</sub> at 40°C. For metabolomic analyses, 90 µL EtOH were added to each sample and aliquots of 50 µL of each extract were used for analyses.

For the analysis of the endo-metabolome of algae after application of aldehydes, an additional extraction step was performed after the first extraction with MeOH/H<sub>2</sub>O. We chose to focus on regulation of the fatty acid pathways, thus targeting fatty acids and their oxidative derivatives such as oxylipins. To separate free fatty acids and their derivatives from polar compounds, samples were first dried under N<sub>2</sub> at 40°C and a second metabolic extraction was made with 200 µL Ethyl acetate and 200 µL H<sub>2</sub>O. The ethyl acetate phase was recovered and dried under N<sub>2</sub> at 40°C. 90 µL EtOH were added to each sample and aliquots of 50 µL of each extract were used for metabolomic analyses in LC-MS.

#### b. Solid Phase Extraction (SPE)

At the end of the treatment step, 50 mL of the surrounding seawater were collected from each sample and diluted in 200 mL distilled water, in which 50 µg of the internal standard 12-OH lauric acid were added. SPE instrument (Dionex Autotrace) is equipped with 6 injection systems designed for 6 SPE cartridges. C18 SPE cartridges (REC18 1 g/6 mL or 500 mg/6 mL) were first conditioned twice with 10 mL MeOH, then equilibrated twice with 10 mL H<sub>2</sub>O. Samples were then entirely loaded on the cartridges. Washing the tubes twice with 10 mL H<sub>2</sub>O removed the most polar compounds from the cartridge and drying them with liquid nitrogen for 15 min permitted to eliminate H<sub>2</sub>O traces in samples. Finally, elution of compounds was performed by 3 injections of 10 mL MeOH on the cartridges. The 8 mL eluates were dried with N<sub>2</sub> and heated at 60°C. They finally were recovered in 200 µL MeOH/H<sub>2</sub>O 80:20 (v:v) and placed in vials for LC-MS analyses.

#### c. Stir-Bar Sorptive Extraction (SBSE)

The extraction by sorption on a stir bar is based on the equilibrium of analytes between the water and a polymeric phase immersed in this matrix, the polydimethylsiloxane (PDMS), supported by a stir bar. Extraction of compounds was carried out during stirring, by sorption of small compounds into the PDMS matrix. Therefore, a SBSE stir bar (GERSTEL Twister) was introduced in the 50 mL of collected surrounding seawater containing a plantlet in the experiment, with 20 ng of the internal standard adonitol, then placed under stirring for 12h. After extraction, stir bars were blotted dry and placed in GC-MS tubes (1 stir bar per tube).

## 2. Metabolomic analyses methods

### a. Gas-Chromatography coupled with Mass Spectrometry (GC-MS)

GC-MS is an analytic method permitting to detect and identify small volatile compounds present in samples, by thermal desorption. This technique may require a previous derivatization of compounds, in order to make them volatile or to stabilize them. Thus, samples were dried under N<sub>2</sub> at 60°C, acetonitrile and Sylon (BSTFA/TMCS 99:1) were added to dry extracts and they were let for 30 – 60 min at 60°C. Solvents were evaporated under N<sub>2</sub> and hexane was added to take the dry extract and analyze in GC-MS.

Metabolites were analyzed by GC-MS using an Agilent GC 7890+ coupled to a 5975 quadrupole mass detector (Agilent, Les Ulis, France) and equipped with a DB/HP-5MS column (methyl phenyl siloxane: 30 m × 0.25 mm I.D. × 0.25 μm film thickness; J&W Scientific, Agilent) in the Electronic Impact (EI) mode at 70 eV. A sample (volume of 2 μL) was injected into the capillary column at 1 mL.min<sup>-1</sup>, with a temperature of injection of 230°C. Then, the temperature gradient used was 60°C for 2 min, 60 to 290°C at 10°C.min<sup>-1</sup> and stabilization at 290°C for 5 min. The range of masses analyzed were from 50 to 800 m/z.

### b. Liquid-Chromatography coupled with Mass Spectrometry (LC-MS)

LC-MS is used to analyze and identify small compounds in a liquid phase, driven by solvents of different polarity. Chemical compounds were first separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an electrospray ionization source (ESI). Scans were collected in both positive and negative ionization modes over a range of m/z 50–1000. Ionization parameters were set as follows: sheath gas 5 psi, auxiliary gas 5 (arbitrary units), sweep gas 0 (arbitrary units), spray voltage 2.7 kV, capillary temperature 300°C, capillary voltage 60 V, tube lens voltage 127 V and heater temperature 300°C. Metabolic samples were analyzed using a 1.9 μm Thermo Hypersil Gold C18 column (100 x 2.1 mm) maintained at 20°C using 5 μl injection volume and a flow-rate of 250 μl.min<sup>-1</sup>. Mobile phase A was composed of 0.1% acetic acid in H<sub>2</sub>O, and mobile phase B was 0.1% acetic acid in acetonitrile. The gradient, starting at 0% B, consisted of an initial hold at 60% mobile phase B from 1 to 3 min, followed by a gradient to 95% B in 12 min and a hold for 4 min, followed by re-equilibration for 4 min at 0% B, for a total run time of 23 min. Xcalibur 2.1 software was used for instrument control and data acquisition.

A specific LC-MS method was developed for analysis of the endo-metabolome of *L. digitata* after exposition to aldehydes. The gradient, starting at 0% B, consisted of an initial hold at 60% mobile phase



B from 1 to 3 min, followed by a gradient to 95% B in 12 min and a hold for 4 min, followed by re-equilibration for 4 min at 0% B, for a total run time of 23 min.

### c. LC-MS/MS

Complementary analyses by LC-MS/MS were performed on samples of the endo-metabolome of *L. digitata* after exposition to aldehydes and on chemical standards, in order to determine the absence or presence of these compounds in the algal samples. These analyses were performed by Yann Guitton in LABERCA (Laboratoire d'étude des résidus et contaminants dans les aliments) in Nantes.

- Chemicals

All solvents and reagents were of analytical LC-MS grade quality. Acetonitrile (ACN), water (H<sub>2</sub>O) and acetic acid were purchased from Sigma-Aldrich. Methanol (MeOH) was purchased from VWR. Standards were leucine-5,5,5-d<sub>3</sub>, L-tryptophan-2,3,3-d<sub>3</sub>, Indol 2,4,5,6,7-d<sub>5</sub>-3-acetic acid and tetradecanedioic-d<sub>24</sub> acid (Sigma Aldrich). The standard mixtures used for the external calibration of the Q Exactive™ were Pierce™ LTQ Velos ESI Positive, for the positive ionization mode, (caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate and Ultramark 1621) and Pierce™ Negative (consisting of SDS, sodium taurocholate and Ultramark 1621) (Thermo Fisher Scientific). Chemical standards tested for their presence in the samples came from Interchim: jasmonic acid, 9(S)-hydroperoxyoctadecatrienoic acid (9(S)-HpOTrE), 13(S)-hydroperoxyoctadecatrienoic acid (13(S)-HpOTrE), 20-hydroxy-leukotriene B<sub>4</sub> and stearidonic acid.

- Instrumentation

A high performance liquid chromatography (HPLC) system (Ultimate® 3000 Dionex, Thermo Fisher Scientific) was used. The chromatographic separation was performed on Hypersil Gold C18 column (2.1 mm × 100 mm, 1.9 μm particle size, Thermo Fisher Scientific). Mobile phase consisted in water (A) and acetonitrile (B) both containing 0.1 % acetic acid (A). The used elution gradient (A:B, v/v) was as follows: 100:0 from 0 to 1.0 min; 40:60 at 3 min; 5:95 at 15 min for 4 min then 100:0 for 4 min. The injected volume was 5 μL, the flow rate was 350 μL min<sup>-1</sup> and the temperature of the column was maintained at 35 °C.

The LC system was coupled to a Hybrid Quadrupole-Orbitrap Mass Spectrometer (Q Exactive™, Thermo Fisher Scientific) with a heated electrospray ionization source (HESI). Nitrogen was used as sheath gas and auxiliary gas at flow-rates of 55 and 10 a.u (arbitrary units), respectively. The ion transfer tube temperature was set at 350°C, the vaporizer temperature at 300°C and the electrospray voltage was set at 3.0 kV in positive mode and -3.0 kV in negative with a s-lens RF level of 50%. For fullMS acquisition a polarity switching ion mode (positive/negative) with a mass range of *m/z* 65-975 at a mass resolving power of 70 000 Full Width Half Maximum (FWHM at 200 *m/z*) are used. The automatic gain

control target and maximum injection time were  $1e6$  counts and 100 ms, respectively. The normalized collision energy (NCE) used for targeted MS/MS and data dependent MS/MS modes was 35. Each target  $m/z$  was monitored with a 40 seconds window (retention time  $\pm$  20 seconds), 2-amu isolation window (target  $m/z \pm 1$  amu).

For injection, the extract was diluted by 2 in ACN:H<sub>2</sub>O (50:50) and 5  $\mu$ L were injected the same day on the LC-HRMS system.

## VI. Bioassays: preference or algal consumption by herbivores

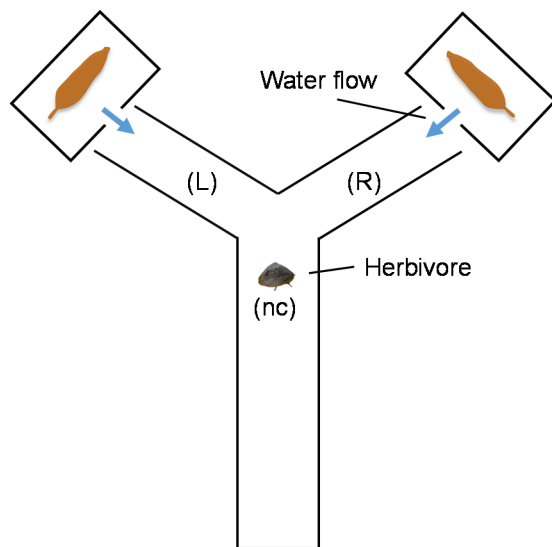
Algal responses to grazing by herbivores were evaluated by the proxy of the consumption of algal tissues by herbivores, following different treatments applied to the alga. Both kelp/herbivore couples were used for bio-assays. Herbivory pressure was regulated according to the size and the number of herbivores on the frond and depending on the size of the frond. *P. pellucida*, much smaller than *S. scurra* or *T. atra*, were put by 5 or 6 on *L. digitata* laboratory-grown fronds, whereas maximum 2 *S. scurra* or *T. atra* were placed on a 20-cm *L. spicata* frond. Two approaches were tested to determine either food preference of the herbivores by choice tests or deterrence by non-choice tests.

### 1. Multiple choice feeding assays

Food preference of herbivores was estimated by attraction to different types of food offered to grazers. Thus, the movement of the herbivore towards one fixed proposition of food allowed to predict potential effects of chemical compounds on herbivore behavior. In each experiment, two food choices were proposed to herbivores.

In the *L. digitata* experiment, food preference of the kelp generalist herbivores abalones (*Haliotis tuberculata*) was tested. Prior to the experiment, abalones of the same age were fasted for 3 days in the experimental system for acclimation. Five tanks, each containing 8 abalones and 4 feeders were installed, with constant seawater and air supply. The photoperiod was 10h30 of light, from 6:30 am to 8:00 pm. As described below, the first food proposition was elicited algae with GG, and the second was healthy algae (without treatment). The first day of bio-assay, in each tank, two feeders were filled with control kelps and the two others with elicited *L. digitata*, 20 g of algae per feeder. The order of elicited and control feeders in each tank was chosen randomly. Consumption of treated algae were measured after 2 and 4 days. Algae were blotted dry and weighted, then replaced in the tank until the end of the experiment. Moreover, during the whole experiment, one infrared camera over each tank filmed 24/24h abalone movements, thanks to animal tagging with phosphorescent paper on their shell.

In the *L. spicata* experiment, choice tests were performed with *T. atra* and *S. scurra*, through a Y tube. Food propositions were situated at the end of the two upper branches of the Y tube, and the herbivore was placed in the lower branch (Fig 14). A seawater flow was created from each food source to the lower branch, in order to permit the herbivore to detect chemical signals coming from the sources and thus going until this emitting source. At the source, water came from 6 L boxes which could contain algae. The food preference of *T. atra* was tested between different treatments (Table 3). The experiment lasted 3 min and was repeated 15 times for each couple of choices, which were then reversed between the left side and the right side of the Y tube.



**Figure 14.** Schema of the Y tube experiment. Food choices were situated at the end of each branch of the Y tube and a seawater flow was created at the food sources. The herbivore was placed in the lower branch and could choose between the left side (L), the right side (R) or the non-choice zone (nc).

**Table 3.** Choices tested in the Y-tube experiment on *T. atra*. In each experiment, 30 *T. atra* individuals were submitted to the choice test. Their choice (choice A, choice B or non choice) and the time of choice were recorded from the moment they show the first movement. C<sub>DG</sub>: Control Direct Grazing, DG: Direct Grazing, IG: Indirect Grazing.

Exp. number	Choice A	Choice B
1	None	None
2	None	C <sub>DG</sub> (Control)
3	C <sub>DG</sub> (Control)	IG (Co-incubated with grazed by <i>T. atra</i> ) <sup>(A)</sup>
4	None	DG (Grazed by <i>T. atra</i> ) <sup>(A)</sup>
5	C <sub>DG</sub> (Control)	DG (Grazed by <i>T. atra</i> ) <sup>(A)</sup>
6	C <sub>DG</sub> (Control)	DG (Grazed by <i>T. atra</i> ) <sup>(B)</sup>
7	DG (Grazed by <i>T. atra</i> ) <sup>(B)</sup>	IG (Co-incubated with grazed by <i>T. atra</i> ) <sup>(B)</sup>
8	C <sub>DG</sub> (Control)	IG (Co-incubated with grazed by <i>S. scurra</i> )
9	DG (Grazed by <i>S. scurra</i> )	IG (Co-incubated with grazed by <i>S. scurra</i> )

Laboratory choice tests were also performed in *L. spicata* to assess the preference of *S. scurra* for natural wounds on stipes. Stipes containing wounds naturally made by *S. scurra* on the field were collected by cutting 15 cm long piece of stipes with a wound on it. In the laboratory, the stipes were individually placed in tanks together with one *S. scurra* individual at a distance from the wound. In parallel, control stipes without grazing marks were cut and placed individually in tanks, with one *S. scurra* put in the middle of the stipe. Herbivore's movement was evaluated, either choosing the wound or another direction. The experiment was repeated 10 times for control stipes and 9 times for wounded stipes. Herbivore's position was noted after 1h and 16h in the first experiment, and after 30 min and 1h in the second experiment.

## 2. Non-choice feeding assays

In non-choice bio-assay, herbivores had only one source of food, directly available for consumption. According to the algal and herbivore species, the number of herbivores on the frond was variable.

For *L. digitata*, laboratory-grown algae were used with its specialized herbivore *P. pellucida*. Bio-assay made after incubation in aldehydes or in GG consisted in placing one young plantlet in 50 mL filtered seawater, exposed to 6 herbivores for 6 days at 13°C. Algal mass was measured before the bio-assay, 3 days and 6 days after exposition to grazers. Five replicates were made for each treatment, while 3 plantlets were used to control autogenic changes of mass during bio-assay.

For *L. spicata*, one frond was cut after treatment and placed in a 6 L box with constant seawater supply and 2 *S. scurra* or *T. atra*. After Grazing or Elicitation treatment, the frond exposed to one of these treatments was cut and used for the bio-assay. Algae were blotted dry and weighted before the experiment, and 2 and 4 days (end of the experiment) after exposition to grazers. Ten replicates were made for each treatment.

## VII. Data processing and statistical analyses

Analyses of the oxidative burst, metabolome modifications and herbivore consumptions led to different types of data, involving specific statistical analyses for each of these types of data. Measurement of H<sub>2</sub>O<sub>2</sub> at different sampling times during 60 min allowed to calculate the total amount of H<sub>2</sub>O<sub>2</sub> accumulated over 60 min. Then, after checking normality with the Shapiro-Wilk test, a 1-way ANOVA was performed, followed by a Student-Newman-Keuls (SNK) post-hoc test to determine the significant differences between each treatment.

Metabolomic data obtained with Xcalibur software corresponded to a chromatogram and mass spectra for each sample. Mass spectra data were processed by Galaxy-Workflow4Metabolomics (W4M) on the online version (Giacomoni et al., 2014), after conversion of raw spectra to mzXML format for LC-MS data and in CDF format for GC-MS data. In W4M, default settings were used for GC-MS data. For LC-MS analyses, data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples necessary at 0.7. For correction of retention time, the obiwrap method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 10. Fillpeaks step was used with the chrom filling method. Finally, annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. The final table contained ions abundances in samples, which corresponded to peak areas. Principal Component Analysis (PCA) and Partial Least Squares – Discriminant Analysis (PLS-DA) were performed with W4M in order to check the quality of the analysis and the distribution of the samples. Then, normalization was done by dividing integrated areas either by the area of the internal standard in the sample when it was detectable, or first by the highest one of the same sample, and then by dividing the areas of one ion by the highest in all the samples. This would allow to compare the relative amount of one ion in different samples. Manual elimination of background and redundant (isotope) ions led to a selection of the most relevant ions which could play a role in metabolomic modifications of algae. Subsequent analyses were performed using the free software R. After installation of the packages vegan, pls and RVAideMemoire, PCA and PLS-DA were performed and Number of MisClassifications (NMC) and p-value of PLS-DA were obtained. Then, using W4M, extraction of the 10 most discriminant ions using VIPs and annotation of some of those ions were made, also from previous studies (Ritter et al., 2008).

Bio-assay results were analyzed differently according to the type of experiment. For non-choice tests, data were often non-normal due to inter-individual variability from variation between algae, between the appetite of herbivores, and also because of the variation in the interaction between the kelp and its herbivore. Thus, to compare mean algal consumption after exposition to different treatments (usually more than 2 treatments per experiment), Kruskal-Wallis tests were performed. For choice tests, multinomial tests were applied for the Y-tube experiment because herbivores had three choices. Food preferences of abalones were analyzed in two ways: Wilcoxon test was applied for comparison between consumption between elicited and control algae.

# Chapter I

Herbivore-induced chemical and molecular responses of the kelps *Laminaria digitata* and *Lessonia spicata*



## Introduction

Kelp forests constitute complex ecosystems that play habitat and food source roles for the diverse associated species. As they are sessile, these brown macroalgae have to cope with biotic stresses, such as grazing by herbivores. During herbivore attack, oligoguluronates are likely to be released at the surface of the kelp and this is well known that kelps respond directly to these MIMPs at different spatio-temporal levels like a transient oxidative burst, metabolic and transcriptomic regulations, and later resistance against some pathogens (Küpper et al., 2002; Bouarab et al., 2004; Weinberger, 2007). However, nothing was known about the nature and the timing of the chemical and molecular responses of kelps upon grazing by herbivores. Therefore, we investigated direct inducible responses of two kelps *Laminaria digitata* from north Atlantic coasts and *Lessonia spicata* from Chile. They were studied at two levels, inside and outside the kelp tissues, in order to assess both compound production and accumulation in tissues and emitted compounds which could be perceived by herbivores or by other kelps. Later on, a part of the emitted compounds could represent a potential signaling for the neighboring kelps to prevent a danger.

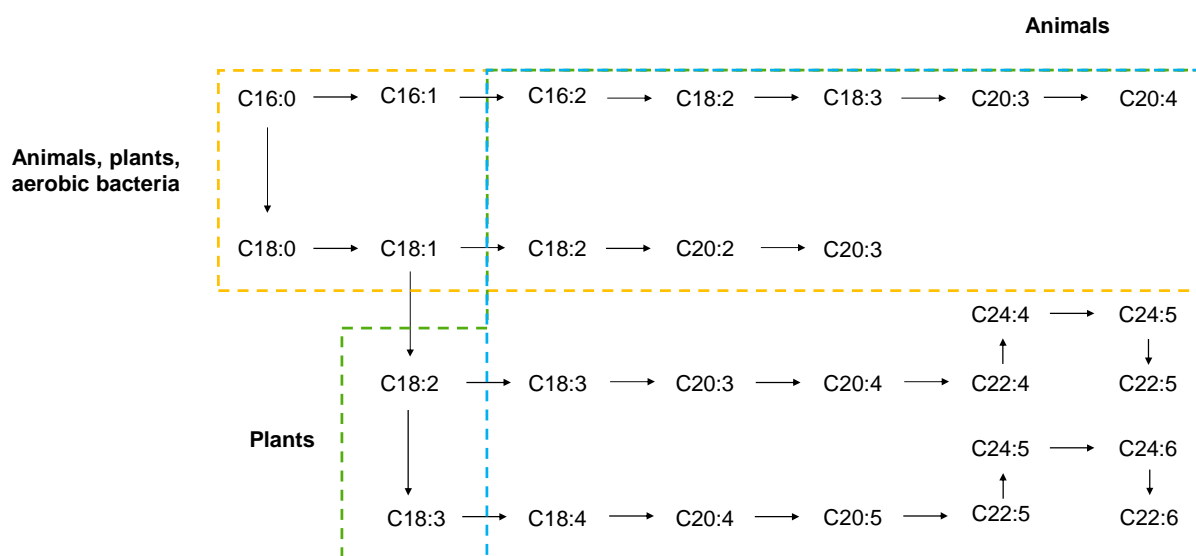
Inner molecular and chemical responses in algae were discussed in the article. Indeed, we already know that, upon challenging with oligoguluronates, the kelp *L. digitata* recognizes this external signal as an elicitor signal, leading to activation of signaling pathways and molecular responses (Küpper et al., 2001; Cosse et al., 2009; Goulitquer et al., 2009). Elicitation generates an oxidative burst as well as a release of polyunsaturated free fatty acids coming from biological membranes, then oxidized and liberated in the medium. Animals and land plants utilize these compounds as stress signaling molecules and brown algae contain fatty acids from both lineages, allowing synthesis of a wide variety of these oxidized derivatives in the brown algae. Simultaneously, defense responses are activated at the transcriptional level, through regulation of genes related to grazing stress, such as in Fucales in which a massive gene regulation is combined with defense responses against herbivores (Flöthe et al., 2014). The objective of this article was to carry out comparative metabolome and transcriptome analyses of the South Pacific kelp *Lessonia spicata* and the North Atlantic kelp *L. digitata* submitted to grazing stress.

My contribution to this article concerned the metabolomic component of this work, with the objective of investigating the metabolic regulations occurring into algal tissues in response to direct grazing stress. It consisted in analyzing the modifications of the endo-metabolome of *L. spicata* and *L. digitata* after exposition to a direct grazing over two days, by comparison to control algae, with metabolomic tools like Liquid Chromatography – Mass Spectrometry (LC-MS) followed by data processing with the developing device Galaxy-Workflow4metabolomics. The experimental design is described in the article and data analysis was processed by two approaches, the first approach consisted in a global analysis of the algal endo-metabolome in order to determine if this metabolome globally changed after a grazing stress. The chemical profiles of some compounds were then investigated in a



second approach, for which we chose to focus on amino acids, fatty acids and oxylipins. Indeed, as previously described, these chemical compounds are involved in algal response to abiotic and biotic stress, and application of the aldehyde 4-HHE which is derived from the fatty acid oxidation activates this pathway (Ritter et al., 2008; Goulitquer et al., 2009). Moreover, as fatty acids in brown algae are related to those in plants and in animals (Fig 15), we can rely on this information to reconstruct the biosynthetic pathways induced in kelps.

In addition to chemical modifications into the alga, we also know that kelps emit chemical compounds in the surrounding medium in response to oxidative stress such as low tide or in response to elicitation by oligoguluronates (Goulitquer et al., 2009). Indeed, C6- and C9-aldehydes were found to be strongly induced and emitted during and 1h after low tide, and their concentrations decreased when the sea rose. However, the quantity changes of the emitted compounds were not monitored over time. We thus investigated chemicals emitted in the surrounding seawater of stressed laboratory-grown *L. digitata* after 1h, 4h, 12h and 24h of elicitation by oligoguluronates, supposed to reproduce a grazing effect. The seawater was collected and extracted both with Solid Phase Extraction (SPE) and with Stir Bar Sorptive Extraction (SBSE) methods, then respectively analyzed in LC-MS in negative and positive ionization modes, and in GC-MS. This complementary approach would allow to have a global vision of the emitted compounds during defense responses and grazing stress.



**Figure 15.** Free fatty acids biosynthetic pathways in plants, animals and aerobic bacteria. From Goulitquer (2008).

## I. Kelp endo-metabolome in response to grazing

### General outline

Global metabolomic analyses resulted in acquisition of 211 ions for *L. digitata* and 818 ions for *L. spicata*, which were considered for statistical analyses. In both kelp species, PLS-DA allowed to discriminate between grazing and control treatments. However, they did not show differences of the metabolome according to time. This could suggest that produced compounds into algal tissues after 6h undergo a concentration stabilization, at least during 48h. Moreover, annotation of 9 free fatty acids (FFA), 3 oxylipins, and 9 amino acids (AAs) was achievable, and the patterns of these targeted compounds showed a clear difference between samples collected after 6 and 12h and those collected after 24 and 48h of grazing. Amounts of amino acids strongly increased at 12h, then reaching control levels at 24h. However, free fatty acids and their oxidized derivatives started to accumulate at 12h, reaching higher levels later than amino acids, at 24h, then decreasing at 48h. Fatty acids released during grazing stress were eicosanoids and octadecanoids, both direct precursors of oxylipins such as prostaglandins, also detected in samples mostly at 24h after grazing. Thus, this work on metabolic regulations in kelp tissues during grazing stress allowed to highlight the activation of metabolic pathways related to accumulation and oxidation of free fatty acids, as well as a release of amino acids in algal tissues.

## Article

### Herbivore-induced chemical and molecular responses of the kelps

#### *Laminaria digitata* and *Lessonia spicata*

Andrés Ritter<sup>1,2,3,Y</sup>, Léa Cabioch<sup>1,2,3</sup>, Loraine Brillet-Guéguen<sup>4,5</sup>, Erwan Corre<sup>4,5</sup>, Audrey Cosse<sup>1,2</sup>, Laurence Dartevelle<sup>1,2</sup>, Harold Duruflé<sup>1,2</sup>, Carina Fasshauer<sup>1,2</sup>, Sophie Goulitquer<sup>1,2</sup>, François Thomas<sup>1,2</sup>, Juan Correa<sup>3,6</sup>, Philippe Potin<sup>1,2</sup>, Sylvain Faugeron<sup>3,6</sup>, Catherine Leblanc<sup>1,2,\*</sup>

<sup>1</sup> Sorbonne Universités, UPMC Univ Paris 06, UMR 8227, Integrative Biology of Marine Models, Station Biologique, Roscoff, France

<sup>2</sup> CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique, Roscoff, France

<sup>3</sup> Centro de Conservación Marina and CeBiB, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

<sup>4</sup> Sorbonne Universités, UPMC Univ Paris 06, FR2424, Analysis and Bioinformatics for Marine Science, Station Biologique, Roscoff, France

<sup>5</sup> CNRS, FR2424, Analysis and Bioinformatics for Marine Science, Station Biologique, Roscoff, France

<sup>6</sup> Sorbonne Universités, UPMC University Paris 06, CNRS, UMI 3614, Evolutionary Biology and Ecology of Algae, Station Biologique, Roscoff, France

\* Corresponding author

E-mail: catherine.leblanc@sb-roscoff.fr

<sup>Y</sup>Present address: Sorbonne Universités, UPMC, Institut de Biologie Paris-Seine, CNRS, Laboratoire de Biologie Computationnelle et Quantitative, 15 rue de l'École de Médecine, 75006 Paris, France.

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## Abstract

Kelps are founding species of temperate marine ecosystems, they evolve in the intertidal coastal areas where they are often challenged by generalist and specialist herbivores. As most of sessile organisms, kelps develop defensive strategies to restrain grazing damage and preserve their own fitness during interactions with herbivores. To decipher some inducible defense and signaling mechanisms, we carried out metabolome and transcriptome analyses in two emblematic kelp species, *Lessonia spicata* from South Pacific coasts and *Laminaria digitata* from north Atlantic, when challenged with their main specialist herbivores. Mass spectrometry based metabolomics allowed to observe large metabolic changes induced in these two brown algae following challenges with their own specialist herbivores. Targeted metabolic profiling also showed that free fatty acid (FFA) and amino acid (AA) metabolisms were particularly regulated under grazing in *L. spicata*. An early stress response was illustrated by the accumulation of Sulphur containing amino acids in the first twelve hours of herbivory pressure. At latter time periods (after 24 hours), we observed FFA liberation and eicosanoid oxylipins synthesis likely representing metabolites related to stress. Transcriptomic analysis identified sets of genes specifically induced by grazing in both kelps. Most of these genes were particularly activated by herbivore stress after 24 hours, suggesting that transcriptional reprogramming could be operated at this time period. We demonstrated the potential utility of these genes as molecular markers for herbivory by measuring their inductions in grazed individuals of field harvested *L. digitata* and *L. spicata*. By unravelling the regulation of some metabolites and genes following grazing stress in two kelps representative of the two hemispheres, this work contributes to provide a set of herbivore-induced chemical and molecular responses in kelp species, showing similar inducible responses upon specialist herbivores in their respective ecosystems.

## Introduction

Brown algae are photosynthetic sessile macro-organisms of high ecological relevance in coastal ecosystems of temperate and polar regions. They belong to the Stramenopile lineage, which diverged early in the evolution from Opisthokonts (including animals, fungi) and Plantae (land plants, green and red algae) and constitute one of the rare eukaryotic lineages to reach complex multicellularity [1]. Little is known about their biology, as they were mostly studied for their taxonomic and ecological diversity. The recent genome sequencing of the filamentous brown alga *Ectocarpus siliculosus* underlined particular molecular features in these organisms to cope with highly variable tidal environment such as an extended set of light-harvesting and pigment biosynthesis genes, and original metabolic processes such as halide metabolism [2]. Brown algae include mostly marine species ranging from small filamentous organisms to large kelps measuring several meters in size. Kelps (order of Laminariales) have primordial roles in rocky coastal ecosystems of temperate regions, from North and South hemispheres, as they form true forests of considerable biomass, hosting highly diversified ecosystems [3]. These marine organisms are also exploited for nutritional or industrial purposes and therefore constitute a significant economical resource. One particularly important question is what makes the ecological and evolutionary success of these giant marine primary producers. Indeed, kelps are continuously submitted to biotic attacks including viruses, bacteria, fungi brown algal endophytes or grazing herbivores. Yet, they are the only taxon that evolved large sizes (i.e. few m to tens of m), somehow escaping or overriding the effect of these attacks. The question is relevant also for the future of the rapidly growing field of kelp aquaculture. Indeed, as it is not possible to spread pesticides in the ocean (i.e. the currents would wash away the products before they actually have any effect in a marine culture), it is necessary to explore methods of protection against herbivore, pests and pathogens that rely on the activation of defense mechanisms by the seaweed itself.

Among biotic pressures, herbivore grazing has a major impact in marine benthic ecosystems [4]. To respond to these challenges, kelps are likely to develop adaptive strategies based on activated and induced metabolic responses [5,6]. Most of the knowledge comes from studies on the north Atlantic kelp *Laminaria digitata*. This organism recognizes biotic threats by the perception of molecular cues such as oligoalginates, recognized as cell wall degradation fragments or lipopolysaccharides related to bacterial pathogens [7,8]. These molecules elicit a signaling cascade resulting in molecular defense responses [8,9]. Elicitor perception generates an early oxidative burst followed by the release of polyunsaturated free fatty acids that are subsequently oxidized into lipophilic signaling molecules grouped under the term oxylipins. In an evolutionary context, this innate immune response in kelps is reminiscent to animals and land plants mechanisms, where oxylipins play pivotal roles as stress-related signaling molecules. Animals predominantly synthesize eicosanoid (C20) derived oxylipins such as prostaglandins, thromboxanes, prostacyclins, leukotrienes or hydro(pero)xy-FA whereas land plants synthesize octadecanoid (C18) derived oxylipins including hydro(pero)xy-FA, divinyl ethers, volatile

aldehydes or jasmonic acid and its derivatives (JAs) [10,11]. In contrast to plants, brown algae retained the capability of synthesizing eicosanoid fatty acids. As a consequence, these organisms synthesize a wide variety of oxylipins such as “animal-like” eicosanoid derived hydro( peroxy)des, epoxides and prostaglandins or “land plant-like” octadecanoid derived aldehydes and the JA precursors 13-HPOTrE or the 12-OPDA [12-14]. The synthesis of oxylipins in brown algae is concomitant to the activation of defense responses at the transcriptional and metabolite level [6, 9, 10]. Moreover, previous results show that the kelp *L. digitata* can recognize the land plant oxylipin, MeJA that triggers defense reactions resulting in the establishment of resistance against invasion by its brown algal endophyte *Laminariocolax tomentosoides* [15]. Furthermore recent analyses demonstrated the existence of elicitor triggered systemic signals in *L. digitata* [16]. Localized oligoguluronate treatment in this kelp generates a systemic signal that induces a distant defense response against the limpet *Patella pellucida*. Interestingly, Chlorpromazine treatment (phospholipase inhibitor) could impair systemic signaling in *L. digitata*, suggesting the implication of FFA oxidation cascade in this process [16].

During interaction with herbivores, induced anti-grazing defenses have already been reported for most of marine macroalgae, including Laminariales [17,18]. However, up to recently, these field or laboratory-controlled experiments were mainly based on herbivore feeding behavior, and few studies have yet explored the molecular and chemical bases of these inducible defense responses in brown algae, at the contrary of land plants. For kelps, although previous studies setup the basis for the study of kelp elicitor-induced defense responses, very few is known about metabolic regulation occurring during grazer attacks. In this context, we carried out comparative metabolome and transcriptome analyses of the South Pacific kelp *Lessonia spicata* and the North Atlantic kelp *Laminaria digitata* challenged with their limpet specialist herbivores to infer about common and specific responses in two kelp families (Lessoniaceae and Laminariaceae).

## Materials and Methods

### Algal material

Juvenile sporophytes of *Laminaria digitata* (Hudson) J.V. Lamouroux (i.e. 5 cm long) and *Lessonia spicata* (Suhr) Santelices (i.e. 20-40 cm long) were collected from the surrounding shores near the Station Biologique of Roscoff (SBR) – France, at the Bloscon site (48°43'31"N ; 3°58'8"O), and at the Estación Costera de Investigaciones Marinas (ECIM), Las Cruces – Chile (33°30'09"S : 71°37'59"O), respectively, and maintained at 14°C in running or filtered seawater (FSW) for 1-3 days before use. Their associated specialized herbivores, *Patella pellucida* Linnaeus in SBR and *Scurria scurra* in ECIM were collected at the same time under kelp beds and kept separately under running seawater until the experiment.

### Controlled grazing experiments

Grazing experiments consisted in co-incubating kelp individuals with their respective grazers: 3 individuals of *P. pellucida* for each *L. digitata* in 50 mL FSW Petri dish and five individuals of *S. scurra* for each *L. spicata* in 10 L of running seawater aquarium at 17°C. Grazing pressure lasted from 6, 12, 24 and 48 hours (3-4 replicates per incubation time, per species). Three to four replicates of sporophytes maintained in absence of limpets in similar experimental conditions were considered as controls for each incubation time. Kelp tissues were then frozen in liquid nitrogen and stored at -80°C.

### Metabolic profiling analyses

Metabolic extracts were obtained from 100 mg of frozen algal powder of *L. digitata* (n=4 except for grazed-48h n=2) or *L. spicata* (n=3 except for control-48h n=2) samples collected after 6h, 12h, 24h and 48h, with 1 mL MeOH:H<sub>2</sub>O (8:2), as already described [19]. For untargeted metabolomic analyses, 1.25 µg of 12-OH-lauric acid was added in each sample, as internal standard. After extraction, aliquots of 50 µL were separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an Electro Spray Ionization (ESI) source at running on the negative mode, as described [19]. Samples were separated using an Acclaim RSLC 120 C18 1.9 µm (2.1 x 100 mm) column 545 (Dionex; Thermo Fisher Scientific, Courtaboeuf, France) maintained at 20 °C using 5 µL injection volume and a flow-rate of 250 µL min<sup>-1</sup> and the mobile phase (0.2% acetic acid in water–acetonitrile) was programmed from 95 : 5 to 5 : 95 acetonitrile–water (v : v).

Mass spectra data were processed by XCMS using the online version of Galaxy-Workflow4metabolomics [20], after conversion of raw spectra to mzXML format. Data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples

necessary at 0.7. For correction of retention time, the obiwarp method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 10. Fillpeaks step was used with the chrom filling method. Finally, annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. Areas for all peaks were normalized using that of the internal standard in the same sample, and normalized areas were used as ion abundances. The multivariate data analysis of relative ions abundances using Partial Least Squares – Discriminant Analysis (PLS-DA) was performed to test for differences in metabolite profiles among grazed and control samples. Further statistical multiple testing analyses were performed to identify the different ions, based on Student T-test and FDR-adjusted p-values. The analysis was carried out on Pareto scaling and log<sub>10</sub>-transformed data using the software SIMCA (13.0, Umetrics, Umeå, Sweden).

For amino acid, free fatty acids and oxylipin targeted profiling in *L. spicata* samples (n=3 except for control-48h n=2), the same extraction and quantification process was applied than for untargeted analyses (see above), adding 1.25 µg of 12-OH-lauric acid and 10 µg of atropine in each sample, as internal standards for negative or positive mode analyses, respectively. Free fatty acid and oxylipin profiles were obtained through negative mode LCMS analyses, and amino acid measurements in positive ion mode. The Hierarchical Clustering analyses of relative abundances changes was carried using Pearson Correlation values in Multi Experiment Viewer 4.9 [19]. Statistical Kruskal-Wallis tests were then performed for each metabolite with grazing stress and time as factors, using the free software R version 3.2.5.

### **cDNA library sequencing and data analysis**

Total RNA was extracted from 100 mg of frozen algal materials according to Apt et al. [21], treated with RNase-free DNase I (Stratagene, La Jolla, CA, USA) to eliminate genomic DNA contamination, and quantified using a Nanodrop ND 1000 spectrophotometer (Labtech International Ltd, Lewes, UK). For each species, two pools of RNA, corresponding to Control or Grazed treatments, were prepared by pooling the same amount of total RNA, extracted from three replicates of control or grazed algal samples, collected after 6h, 12h, 24h and 48h for *L. digitata* (Ld) and after 6h, 12h and 24h for *L. spicata* (Ls). The four non-normalized shot-gun cDNA libraries, namely Ld-Control, Ld-Grazed, Ls-Control and Ls-Grazed, were constructed using the SMART cDNA library kit from Clontech (CA, USA), differentially-labelled and sequenced using 454 sequencing technology (Roche, Branford, CT, USA) at the Max Planck Institute of Berlin-Dahlem.

Raw sequencing data were deposited in the SRA database at NCBI (accessions SRR4149283 for Ld-Control, SRR4159455 for Ld-Grazed, SRR4240918 for Ld-Control, SRR4240919 for Ls-Grazed sequences). Raw reads were cleaned by removing adaptor sequences, empty reads and filtering reads with poor quality using SeqClean (<https://sourceforge.net/projects/seqclean/files/>). For each species, the cleaned 454-generated reads for all sequences (derived from Control and Grazed libraries) were



assembled into non-redundant unigenes using Newbler v2.0.01.14 v2.5 (<http://www.my454.com>), by including cDNA sequences of the two species available in public databases, to increase the quality of clustering. The unigenes sequences are available upon request. Candidates of differentially-expressed unigenes were identified according to their respective number of reads into the two libraries, using IDEG6 web tool [22], based on statistical tests [23]. The putative function of these putative differentially-expressed sequences was analyzed using Blastx and Blastn against the nr database (March 2016).

### **Validation of differentially-expressed genes by qPCR analysis**

To validate the molecular markers of grazing stress, total RNA was extracted from 100 mg of frozen algal material of *L. digitata* or *L. spicata* sampled during laboratory-controlled grazing kinetics or in natural populations. For *L. digitata* grazing laboratory kinetics, the RNA samples were those used in EST analyses, whereas for *L. spicata*, a new experimental grazing experiment was conducted up to 48 hours, corresponding to a new set of RNA samples for both control and grazed algae. For *in situ* survey, grazed and un-grazed fronds from adult sporophytes of *L. digitata* and *L. spicata* (n = 7-8) were sampled at the same locations at Roscoff and Las Cruces (see above and Fig S1). Field samples were immediately flash frozen in liquid nitrogen and kept at -80°C before RNA extraction. Gene expression was then analyzed by qPCR as described by Cosse et al. [9], using the primers sequences provided in S1 Table. Gene transcript level was normalized to the geometrical mean of two reference gene transcript levels in the same sample (tubulin and EF1a genes for *L. digitata*; EF1a and RPL36 genes for *L. spicata*). The Hierarchical Clustering analyses of gene expression changes was carried out on Pearson Correlation values in Multi Experiment Viewer 4.9 [19]. A two-way analysis of variance (ANOVA) with grazing stress and time as categorical predictors was applied to qPCR expression data. All tests were performed using Statistica 7 (Statsoft, Tulsa, OK, USA).

## **Results**

### **Specialist herbivores induced large metabolome alterations in both Chilean and European kelp species.**

Metabolomic profiling by LCMS in the negative mode of grazed algae provided 211 ion peaks in *L. digitata* and 818 in *L. spicata*, that were considered for multivariate analysis (S2-S3 Tables). Partial least squares discriminant analysis (PLS-DA) of these datasets for *L. digitata* explained ~50% of the observed variation. In particular, the axis t[1] discriminated two groups of grazed and control individuals (Fig 1A). For *L. spicata* datasets, PLS-DA analysis explained 80% of the observed variation. Grazed and controlled individuals were distinctively grouped along t[2] axis that explained 54% of the observed variation (Fig 1B). Although multivariate metabolome analysis succeeded to discriminate between

control and grazed kelps, PLS-DA analyses failed to distinguish a general pattern according to time in both species. This result suggests the establishment of a metabolic response from 6h of grazing exposure, which would persist over time of the experiment until 48h. Moreover only a small proportion of ions showed a significant change between the two treatments (T-test and FDR tests,  $p$ -value $<0.05$ , 12 ions over 211 ions for *L. digitata* and 15 over 818 ions for *L. spicata*; see S4-S5 Tables).

### **Metabolic profiling of grazed *L. spicata* highlighted distinctive accumulation patterns of Amino Acids and Free Fatty Acids.**

We previously demonstrated the regulation of free fatty acid (FFA), oxylipin and amino acid (AA) metabolisms by biotic and abiotic stress in brown algae [7,12,19]. Accordingly, we profiled these metabolites in *L. spicata* to evaluate their possible functions in grazing stress defense. LC-MS analysis of extracts obtained from grazed and control individuals of *L. spicata* identified 9 FFA, 3 oxylipins, and 9 AAs. Hierarchical Clustering Analysis of metabolite accumulation in grazed algae compared to controls made a clear distinction between early harvested samples at 6h and 12h opposed to late harvested samples at 24h and 48h (Fig 2A). Moreover, statistical analysis showed a significant induction effect of grazing stress (Kruskal-Wallis test;  $P < 0.05$ ) for 12 of the 21 profiled metabolites (Fig 2A; S6 Table).

Free AAs accumulated early upon grazing, with a 17-fold increase in total detected AA content after 12h of grazing compared to control. This was followed by a drastic drop in AA content, reaching control levels on longer stress periods from 24h onwards (Fig 2A-B). Specifically, 12h of grazing pressure increased the concentration of methionine (34-fold), and its precursors, cysteine (16-fold) and aspartate (16-fold). The AA proline (14-fold) and its precursor glutamate (16-fold), but also valine (14-fold), leucine (15-fold) and isoleucine (9-fold) showed a similar pattern of induction with a peak after 12 hours of grazing stress compared to controls (Fig 2B).

In contrast to AAs, FFA and FA-derivatives accumulated at latter stress periods, and 10 out of the 12 profiles presented a significant induction by grazing stress (Fig 2A; S6 Table). This difference in timing was clearly observed in the Hierarchical Clustering Analysis of metabolite accumulation (Fig 2A). Most of the detected FFA and FA-derivatives accumulated from 12 hours of grazing to peak at 24h and then dropped at 48h (Fig 2C-D). Remarkably, grazing stress induced a strong liberation of FFAs from both octadecanoid and eicosanoid pathways. Octadecanoic acid (C18:0) was the most highly accumulated FFA upon grazing stress showing 10-fold increase at 24h (Fig 2A;C). Linoleic (C18:2), linolenic (C18:3) and arachidonic (C20:4) acids were the most highly accumulated desaturated FFAs and showed increases of 5-6 folds at 24h of grazing stress compared to control conditions (Fig 2A;C). These FFAs are direct precursors of oxylipins. Accordingly, we detected significant accumulations of the cyclopentenone prostaglandins J2, A2 and B2 under grazing stress. These oxylipins co-accumulated

with their precursor arachidonic acid, increasing after 12h of grazing stress, reaching a peak at 24h and then decreasing up to control levels at 48h (Fig 2A;D).

### **EST analysis identified differentially expressed genes under grazing challenge in kelps**

We next examined transcriptome variations of grazed *L. digitata* and *L. spicata* to identify particular genes or eventual processes related to herbivory in kelps. In this purpose, juveniles of *L. digitata* and *L. spicata* were challenged or not with their respective specialist grazers up to 48 or 24 hours, respectively, and an EST approach was developed using alga sampled along the experimental time-courses. A total of 444,474 reads were produced with an average length of 184 nucleotides (Table 1). After assembly, 15,454 and 16,511 unigenes (singletons + contigs) were retrieved for *L. digitata* and *L. spicata*, respectively. Among these, only a small fraction (0.8%) showed significant ( $P < 0.01$ ) differential expression between grazing and control treatments. (Fig 3A; S7-S8 Tables). Namely, 64 unigenes showed significant up-regulation and 58 unigenes were down-regulated in grazed *L. digitata* (S7 Table). *L. spicata* analysis resulted in 64 up-regulated and 70 repressed unigenes upon grazing (S8 Table).

Blastx and Blastn analysis of differentially expressed sequences against the nr database retrieved little homology with gene databases, underlying their probable specificity to brown algal sequences (S7-S8 Tables). In many cases (i.e. 97/122 for *L. digitata* and 84/132 for *L. spicata*) Blast results retrieved non coding sequences, likely corresponding to 3'UTR sequences of the cDNA library. Indeed the brown algal genes have been shown to feature a very long 3'UTR [2], leading to their over-prevalence in cDNA libraries.

To confirm results obtained from *in silico* analyses of EST libraries, we selected 15 unigenes in *L. digitata* and 13 in *L. spicata* among the top up-regulated expressions under grazing stress (S7-S8 Tables). Expression patterns of these unigene candidates were followed in juvenile individuals of both species challenged with their specific limpet grazers *P. pellucida* and *S. scurra*. Samples of both species were harvested after 6, 12, 24 and 48 hours of grazing challenge for RNA extraction.

The qPCR expression analysis in *L. digitata* showed a significant induction effect (2-way ANOVA;  $P < 0.05$ ) of grazing stress in 14 out of the 15 tested unigenes (Fig 3B; S9 Table). The 2-way ANOVA test also found some significant effects ( $P < 0.05$ ) of time, meaning that expression of 12 of them varied significantly over time. Furthermore most of the tested unigenes presented significant interactions between time and grazing factors ( $P < 0.05$ ), meaning that the up-regulation of 10 genes was dependent on a particular time (S9 Table). The relation between grazing and time was represented by a hierarchical clustering analysis of unigene fold-change expression of grazed compared to control algae in Fig 3B. This analysis identified a large group of 10 unigenes showing an expression pattern of induction at 24-48 hours of grazing stress. The remaining 5 unigenes showed rather rapid induction, or significant downregulation.

We used the same validation approach in *L. spicata* by testing 13 unigenes that showed strong induction by grazing stress in the EST library. The qPCR results of unigene expression were then analyzed through 2-way ANOVA test using as factors grazing treatment and time. This analysis identified 8 out of 13 unigenes as significantly different ( $P < 0.05$ ) between control and grazed groups (Fig 3C; S10 Table). In less cases than for *L. digitata*, 2-way ANOVA test also found some significant effects ( $P < 0.05$ ) of time and its interaction with grazing stress, suggesting that the induction of one gene is dependent on a particular timing. Hierarchical clustering analysis grouped together 6 out of the 13 genes being significantly affected by grazing, time and the interaction between these factors (namely Ls\_contig02470, Ls\_contig00344, Ls\_contig01374, Ls\_contig02225, Ls\_contig00467, Ls\_contig00239, Fig 3C). These genes showed expression patterns increasing from 6h to peak at 24h of grazing stress following a drop of induction at 48h (Fig 3C).

### **Field validation of grazing stress markers**

We further evaluated whether the identified up-regulated sequences could serve as markers of grazing exposure in the field. With this purpose, *L. digitata* adult fronds grazed by *Patella pellucida* (Fig. S1) were harvested at the shore of Roscoff – France together with ungrazed individuals as negative controls. RNA was then extracted from individuals to quantify expression of 8 unigenes (*i.e.* Ld\_contig02543; Ld\_contig02608; Ld\_contig02668; Ld\_contig02406; Ld\_contig02587; Ld\_contig02511; Ld\_contig00126; Ld\_contig00071) that were previously validated in laboratory grazed conditions (Fig 3B). The results showed significant transcript accumulation of only three unigenes, Ld\_contig02543, Ld\_contig02587 and Ld\_contig00071, in grazed adult individuals compared to un-grazed algae collected in the field (Fig 4A).

Similarly, adult fronds of *L. spicata* grazed by *S. scurra* were harvested at the shore of Las Cruces – Chile (Fig. S1) together with ungrazed individuals as negative controls. RNA was then extracted from individuals to quantify expression of 7 unigene sequences (Ls\_contig01083; Ls\_contig00239; Ls\_contig01374; Ls\_contig00467; Ls\_contig02225; Ls\_contig02470 and Ls\_00344) presenting high confidence up-regulation in laboratory conditions (Fig 3C). All tested unigenes showed significant up-regulation in grazed individuals compared to controls. Altogether, these results have validated a number of grazing molecular markers in *L. digitata* and *L. spicata*, demonstrating their strong activation by grazing regardless of developmental stages or laboratory set-up conditions.

## Discussion

Our metabolome analysis succeeded to make global distinctions between grazed and control metabolomes of *L. digitata* and *L. spicata* (Fig 1). This result suggests that these two kelps are able to recognize their specialist herbivores, and regulate their metabolism, according to grazing challenge. Moreover, metabolite profiling (Fig 2) revealed that grazing induces in *L. spicata* early and late responses, implicating an early accumulation of aminoacids, followed by the later liberation of FFA and the synthesis of prostaglandins.

In *L. spicata*, significant accumulations of AAs were detected in grazed individuals during the first six hours of stress and peaked after 12h. Sulphur containing AAs (SAAs) such as Methionine and its direct precursors Cysteine and Aspartate were rapidly and highly accumulated under herbivore attack, suggesting their functions in grazing stress. Indeed SAAs play key roles in protein synthesis and function, and these Sulfated groups AA are also particularly sensitive to oxidation caused by stress. In this context high accumulation of SAAs could be explained by oxidative stress conditions generated by grazer attack. This hypothesis is supported by previous transcriptome analysis of *L. digitata* elicited with oligoguluronates (GGs) [9]. GGs is a kelp cell wall digestion product constituting a microbe-associated molecular pattern. *L. digitata* associates the presence of GG with a biotic attack, thereafter generating an oxidative burst cascade resulting in the transcriptional activation of defense responses [6,9]. Results of this analysis identified as induced a Methionine Sulfoxide Reductase (MSR) gene. MSRs are enzymes that act to reduce sulfoxide groups generated in Methionine residues by oxidative stress, therefore restoring protein function. In relation with antioxidant components, Glutamate contents were also highly increased by grazing in *L. spicata*. This AA constitute building block of glutathione that plays a key role as anti-oxidant in both biotic and abiotic stress mechanisms in eukaryotes [24]. This tripeptide is used by land plants and brown algae as one of the main antioxidant component in the Ascorbate-Glutathione (ASC-GSH) cycle [25,26]. Beside its function as compatible osmolyte, proline contributes to stabilizing sub-cellular structures (e.g., membranes and proteins), scavenging free radicals thus buffering cellular redox potential under stress conditions in land plants [27]. Moreover, exogenous proline application upregulates the activities of enzymes in the ASC-GSH cycle in plants [28]. It has also been proposed that its accumulation may be part of plant stress signal influencing adaptive responses. Previous studies in *E. siliculosus* determined this AA as particularly accumulated during short-term response to oxidative and osmotic stress [29]. Altogether, the observed increased contents of these AAs is likely to respond to the alteration in the redox cellular status following herbivore attack.

FFA and oxylipins contents were also differentially accumulated by grazing stress in *L. spicata*. In contrast to AAs, the accumulation dynamics of these compounds increased from 12h to peak at 24h then decreased at 48h of grazing. This timing is in accordance to our previous observations of FFA and oxylipin biosynthesis in *L. digitata* following abiotic stress conditions [12]. Grazing stress induced in *L.*

*spicata* significant accumulations of both octadecanoid and eicosanoid FFAs together with eicosanoid derived oxylipins. Similar results of FFA and oxylipin accumulations were observed in *L. digitata* following bacterial lipopolysaccharide induction, which was recognized by the kelp as a PAMP [7]. Animals, plants or red algae react to biotic attacks by the activation of signaling reactions which include oxidative bursts and oxylipin formation from either octadecanoid (land plants), eicosanoid (animals) or both (red algae) pathways such as jasmonic acid or prostaglandins [30,31]. In agreement with the functions of FFAs and oxylipins, it was previously shown that arachidonic acid or methyl-jasmonate could trigger an oxidative burst in *L. digitata*, which induced resistance against the infection of the endophyte pathogen *Laminariocolax tomentosoides* [15]. In this context, the observed accumulations of FFAs and prostaglandins in *L. spicata* in response to grazing stress likely constitute molecular cues triggering defense reactions against *S. scurra*. Grazing in *L. spicata* stress induced the synthesis of PGA<sub>2</sub>, PGB<sub>2</sub> and PGJ<sub>2</sub> oxylipins that derived from arachidonic acid. In agreement, previous observations in *L. digitata* showed that only arachidonic acid but not linoleic acid could trigger defense response against pathogen infection in this kelp [15]. Altogether, these results suggest the preponderant importance of eicosanoid derived metabolites to trigger defense responses during seaweed-herbivores interactions.

Environmental abiotic and biotic stresses (such as wounding or pathogen attack) in plants can trigger the stress defensive mechanisms through massive reprogramming of gene expression. Our transcriptome analysis attempted to evaluate if a 2 days-long grazing challenge could induce large gene expression changes in kelps and succeeded to identify as differentially expressed only a reduced number of unigenes (122 and 132 for *L. digitata* and for *L. spicata*, respectively; Fig 3), representing 0.8% of the total identified unigenes. It's likely that our results don't accurately reflect whole transcriptome changes operating in kelps under grazing stress, over this short term period. However, recent transcriptomic analysis of *F. vesiculosus* grazed by the adapted specialist herbivore, *Littorina obtusata*, for 3 days revealed 61 up- and 124 down-regulated genes, the stronger gene regulation occurring between 2 and 3 weeks of grazing (241 and 270 genes differentially expressed at day 15 and 21, respectively) [32]. Moreover, we found very few homologies between our unigene library and known gene functions. This results could reflect the reduce sequence length coverage of our unigene libraries [2], but also partly underlines the paucity of reliable gene annotations for brown algal sequences. Most of the identified differentially expressed genes in both kelp species showed a strong peak of induction after 24 and 48 hours of grazing stress, suggesting their implication in stress responses. Remarkably, this result is in co-occurrence with our observed oxylipin synthesis in *L. spicata*, suggesting a possible link between oxylipin accumulation and the defense responses. Nevertheless further experiments are needed to confirm their direct activation by FFAs or prostaglandins. Future gain of genomic information will allow to identify functions of these interesting genes, for which strong relatively early inductions observed under grazing support their implication in activation of anti-herbivory adaptive responses.

An additional line of evidence supporting the implication of the identified unigenes in grazing stress was obtained from our field experiments. This approach was particularly successful in *L. spicata*, but not in *L. digitata*. In this later kelp, the up-regulated genes, validated in laboratory conditions but not *in situ*, might correspond to very early responsive genes (up to 2 days of grazing) or be rather related to the experimental setup and to early developmental stages, as young sporophytes were used. Indeed the timing of grazing pressure in the field samples was unknown and could therefore exceed 2 days. Interestingly, in *L. spicata*, the herbivore *S. scurra* generated large wound zones where our identified unigenes showed high expression. Moreover the high induction of these genes in recurrently grazed fronds further suggests the association of these sequences with the setup of persistent responses to grazing. Recent studies demonstrated the ability of *L. digitata* to vehicle systemic signals inducing defense reactions distantly of elicited zones [16]. GG elicitation generated an oxidative burst signal that was transmitted systemically through the kelp tissue, activating defense genes in a process that is likely dependent on oxylipins signaling. Moreover, authors observed reduced grazing activity of *P. pellucida* distantly from elicited areas. These studies are reminiscent to recent findings in land plants showing distant propagation jasmonate signaling triggering defense distantly of wounded areas [33]. In line with these results it would be interesting to assess if the identified unigenes can be activated by oxylipins signaling pathways distantly to grazed areas.

## Conclusion

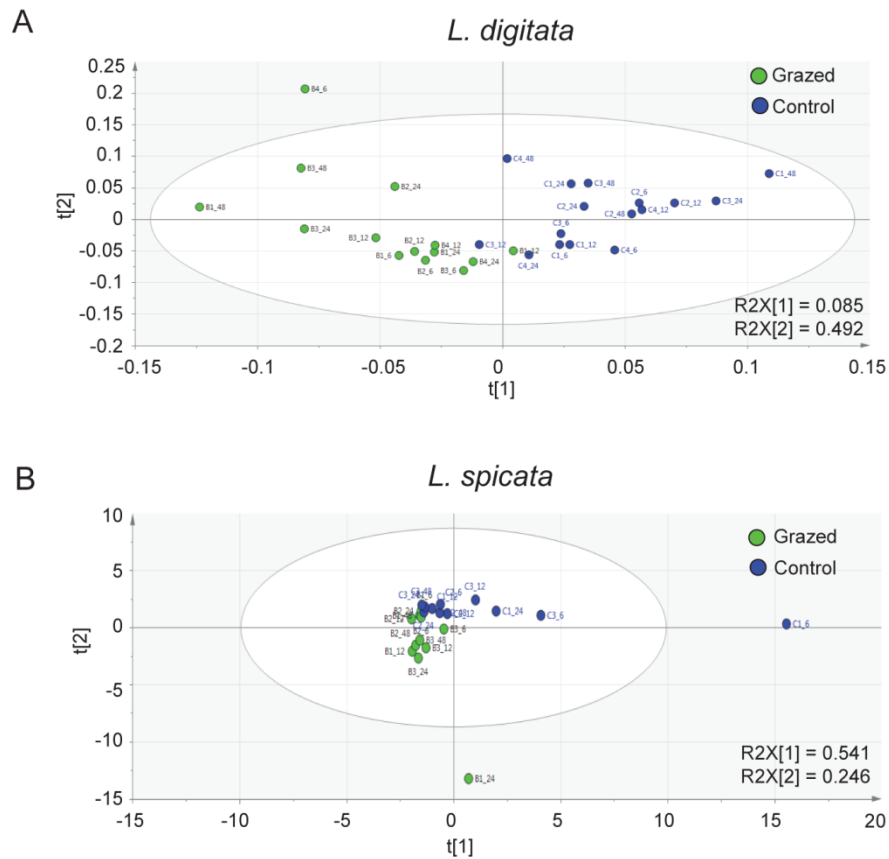
Using laboratory-controlled bioassays we have shown that grazing by specialist herbivores induces both significant modification of the kelp metabolome and specific gene regulation. In addition, our metabolite profiling results show that *L. spicata* responds to herbivore attack by a rapid response following the first hours of stress consisting in the accumulation of AAs likely constituting an antioxidant response. This response is followed by a response consisting in the synthesis of FFAs and oxylipins which may act as signaling cues to trigger latter immune responses against herbivores. Finally our results determined a set of genes related to grazing stress in field populations of marine kelps. These genes could be used as markers to study the functional dynamics of seaweed-herbivore interactions in natural populations or as selection markers *i.e.* in the context of kelp breeding programs. Recent developments in next generation sequencing technologies open exiting perspective of this work. Genome Wide Association Studies of grazed kelp populations might provide more precise traits of evolution related to herbivore defense in these organisms.

## **Acknowledgements**

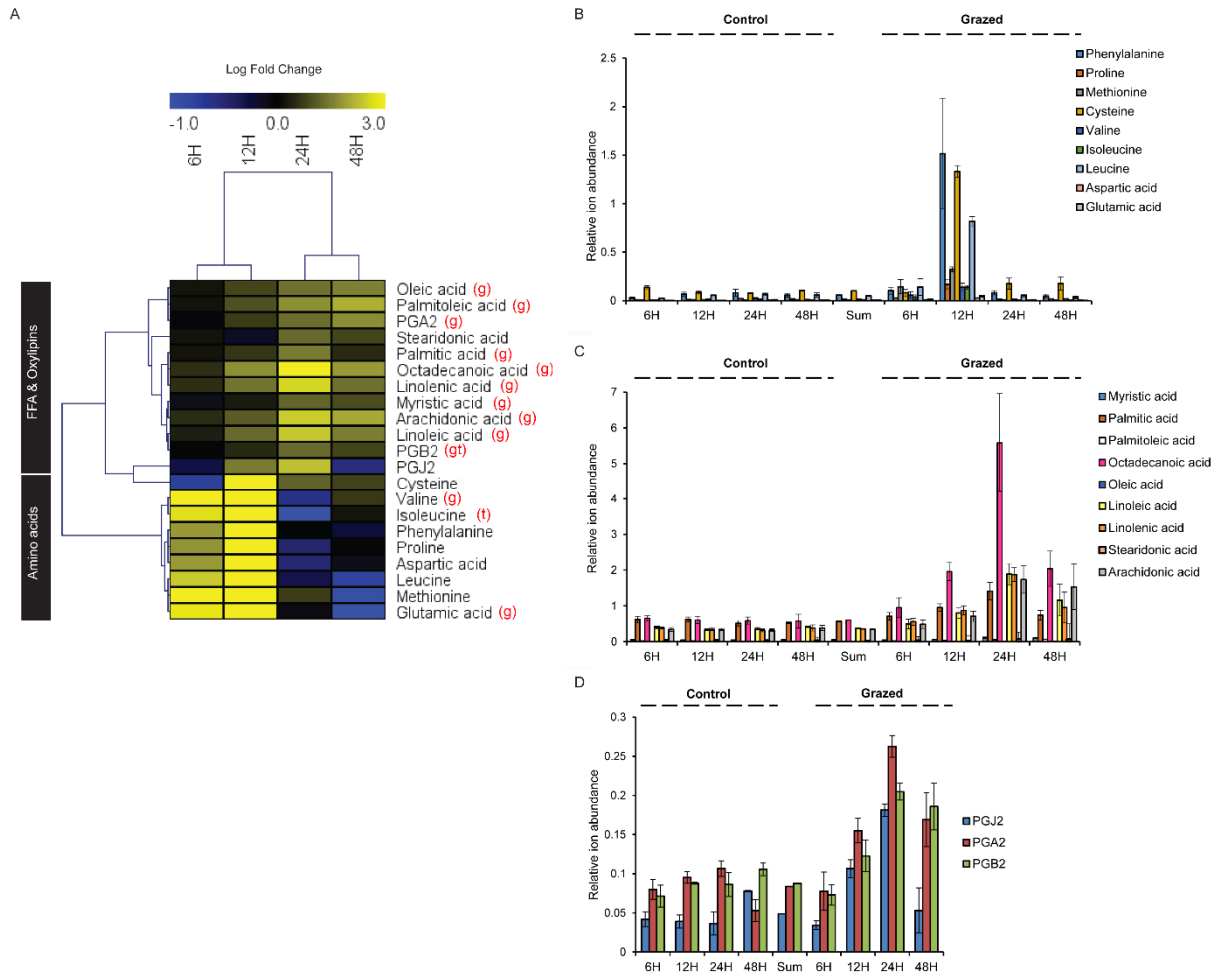
This work was supported by the 'Centre National de la Recherche Scientifique' and by the 'Université Pierre et Marie Curie, Paris 06'. The project was also partly founded by the IDEALG project (grant ANR-10-BTBR-04-02), and by Region Bretagne (L. Cabioch's PhD allocation no. J13U220/ARED SIGNALG). The Las Cruces experimental access was supported by the European Community through the ASSEMBLE programme no. 227799. We are very grateful for assistance and technical help of Karine Cahier during mass spectrometry data collections on MetaboMer core facility at the Station Biologique de Roscoff.



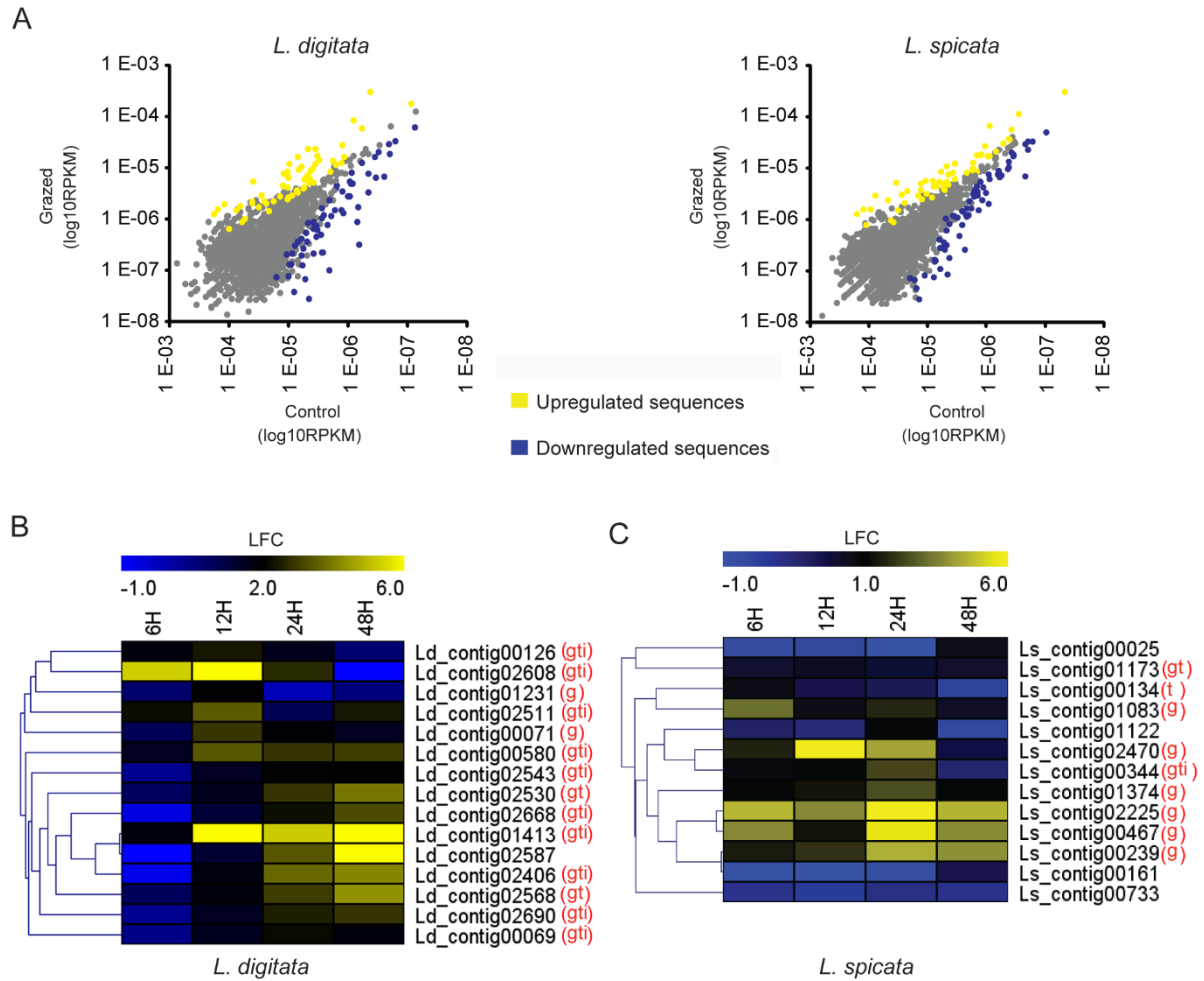
## Figure legends



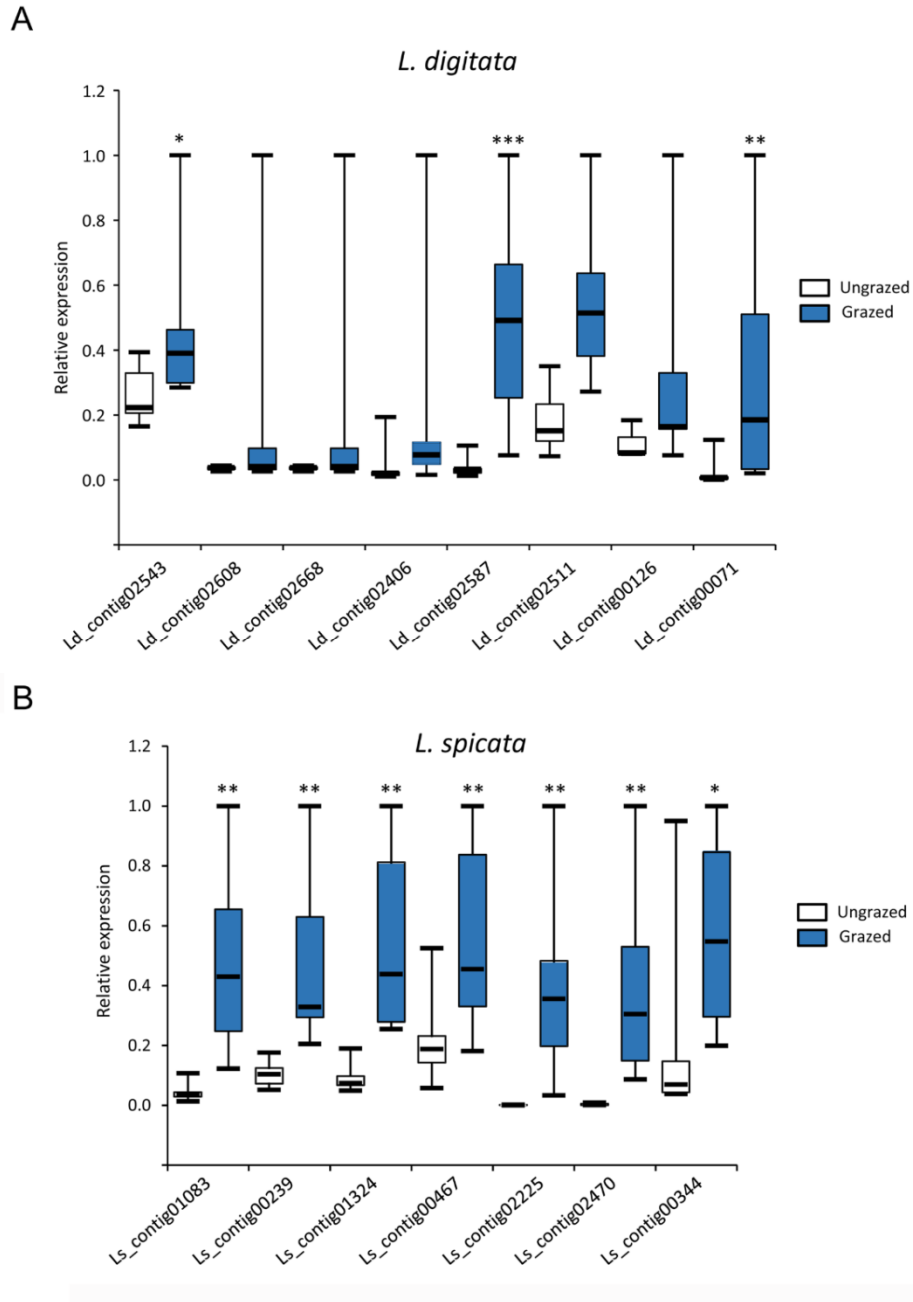
**Fig 1. Specialist herbivores induced large metabolite variations in kelps.** PLS-DA score plots of metabolite ions detected by LC-MS/MS in *L. digitata* (A) and *L. spicata* (B) juveniles under controlled conditions (211 and 818 monoisotopic peaks respectively). Blue spots represent control algae and green spots represent grazed algae.



**Fig 2. Metabolic profiling in grazed *L. spicata* highlighted distinctive accumulation dynamics of Amino Acids and Free Fatty Acids.** (A) Heat map of the amino acids, free fatty acids and oxylipins detected in *L. spicata* in laboratory controlled conditions. Values represent the log fold-change of control conditions ( $n = 3$ ). Time points and compounds were clustered according the Pearson Correlation. The letter (g) indicates a significant difference according to grazed condition, and (t) indicate significant differences for time ( $P < 0.05$ , Kruskal-Wallis test). (B-D) Time course of relative abundances of free amino acid (B), free fatty acids (C) and oxylipin contents (D) in control and grazed *L. spicata* (mean of three replicates  $\pm$  SE).



**Fig 3. Unigene libraries of grazed *L. digitata* and *L. spicata* revealed differentially expressed transcripts.** (A) Scatter-plot representations comparing Grazed and Control unigene libraries of *L. digitata* and *L. spicata*. Values are expressed as Reads Per Kilobase of transcript per Million of total reads (RPKM). Yellow dots represent significantly up-regulated unigenes whereas blue dots significantly down-regulated unigenes. (B-C) Heatmaps showing the qPCR validation of grazing markers in laboratory controlled conditions. Unigenes are presented according to a hierarchical clustering analysis. Values are represented as log fold-change (LFC) of control conditions (n=3). The letter "g" indicates a significant difference according to grazed condition, "t" indicate significant differences for time and "i" indicates the significant interaction between grazing and time ( $P < 0.05$ , 2-way ANOVA).



**Fig 4. Validation of grazing marker genes in natural kelp populations of *L. digitata* and *L. spicata*.** (A-B) Whisker-plot representations of qPCR expression values from candidate grazing marker sequences in ungrazed (white) or grazed (blue) natural populations of *L. digitata* and *L. spicata* (n= 7-8). Expression values were normalized to a maximum of 1. Continuous horizontal bars along boxes represent the median values. For each marker, the effect of grazing was tested using U-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

**Table 1. General features of 454-generated ESTs libraries**

	<i>Laminaria digitata</i>	<i>Lessonia spicata</i>
# Total reads	201664	242810
# Total ESTs (after cleaning)	104479	126002
# Unigenes (after assembling)	15454	16511
Size range (nt)	50-2817	50-1335
Average length (nt)	187	181
# Contigs	2676	2716
Average length (nt)	358	361
# Singletons	12778	13795
Average length (nt)	151	146

## Supporting Information

**S1 Fig.** Representative pictures of *Laminaria digitata* (A) and *Lessonia spicata* (B) with their respective specialist herbivores *Patella pellucida* Linnaeus (A) and *Scurria scurra* (B), showing typical grazing damages on algal tissues. Photos by LC, FT, CF, SF and CL.

**S1 Table.** Primers used in the qPCR analysis study.

**S2 Table.** List of ions identified in *L. digitata* and relative abundance peaks in control and grazed individuals at 6, 12, 24 and 48h of stress.

**S3 Table.** List of ions identified in *L. spicata* and relative abundance peaks in control and grazed individuals at 6, 12, 24 and 48h of stress.

## Provided online

**S4 Table.** Statistical analysis comparing metabolite ions of grazed vs. control individuals of *L. digitata*.

**S5 Table.** Statistical analysis comparing metabolite ions of grazed vs. control individuals of *L. spicata*.

**S6 Table.** Statistical analysis comparing amino acid, free fatty acids and oxylipin relative profiles of grazed vs. control individuals in *L. spicata*.

**S7 Table.** Differentially expressed unigenes under grazing stress identified by sequencing analysis of the *L. digitata* cDNA libraries.

**S8 Table.** Differentially expressed unigenes under grazing stress identified by sequencing analysis of the *L. spicata* cDNA libraries.

**S9 Table.** Validation of differentially expressed unigenes under grazing stress by qPCR analysis in *L. digitata*.

**S10 Table.** Validation of differentially expressed unigenes under grazing stress by qPCR analysis in *L. spicata*.

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**Fig. S1**

**A)**



**B)**



**S1 Fig.** Representative pictures of *Laminaria digitata* (A) and *Lessonia spicata* (B) with their respective specialist herbivores *Patella pellucida* Linnaeus (A) and *Scurria scurra* (B), showing typical grazing damages on algal tissues. Photos by LC, FT, CF, SF and CL.

**S1 Table.** Primers used in the qPCR analysis study.

Primer name	Sequence	Tm	Product Length
Ld_EF1a_fw1q	GGCCGCTTGTGCTGATC	60	71
Ld_EF1a_rev1q	GGAACAGAGGAGAGAAGGAACGA	60	
Ld_Tuba_fw1q	TCGTGCAGTTGGGTTTCGA	60	62
Ld_Tuba_rev1q	CAAGACGCCATGCGATACG	60	
Ld_Act_fw3q	GGATGTCCGCCACGATCT	59	87
Ld_Act_rev3q	ACCTGACGGACAACCTGATGA	59	
Ld_contig02587_PCRQ_F	CGCCGTGCTCCCTGTTCC	59.8	120
Ld_contig02587_PCRQ_R	GCCCAATCCCCTGCTGTAATAAAG	60.2	
Ld_contig02406_PCRQ_F	TGCCATAGGGGTAAGTGCTGTAG	60	80
Ld_contig02406_PCRQ_R	AGTAGTTCCTCCGTTGAGAAGCC	59.6	
Ld_contig02608_PCRQ_F	GGATGATGATGCTGCTGCTTGTG	60.3	142
Ld_contig02608_PCRQ_R	ACCCTAGAGGTATGTGTGAGTGC	59.7	
Ld_contig02511_PCRQ_F	GGGACGCAGCAGTACGC	59.9	132
Ld_contig02511_PCRQ_R	GCCGACGATCAAGCCAAC	59.7	
Ld_contig00580_PCRQ_F	TGTGAAGTGTGGCGAGAACC	60.1	149
Ld_contig00580_PCRQ_R	ATACCTGCCTGGCTTATCATCCTG	59.9	
Ld_contig02543_PCRQ_F	GCAGTAGTACCCITCACTAAATCCG	59.5	93
Ld_contig02543_PCRQ_R	AGGTGATGCTTGTGTTGAATGAGG	59.4	
Ld_contig01231_PCRQ_F	AGCGGAAGATGGAACCTGAGATTCG	60.6	63
Ld_contig01231_PCRQ_R	GTTCAAGGAAATTCCTCGTGTATG	59.8	
Ld_contig00069_PCRQ_F	GCAGTAGTACCCITCACTAAATCCG	59.5	93
Ld_contig00069_PCRQ_R	AGGTGATGCTTGTGTTGAATGAGG	59.4	
Ld_contig02568_PCRQ_F	GTGGCAACAAGAAAGAGAAGAGAG	59.8	119
Ld_contig02568_PCRQ_R	CGGAAACCCTAAGGACGATATGG	59.8	
Ld_contig00071_PCRQ_F	TGGTCAACTAACGCTCCCTCC	59.7	136
Ld_contig00071_PCRQ_R	CTCGCCACAACAGCACTACATTC	60	
Ld_contig02690_PCRQ_F	ACTACAACCTTAAACGAGCAACAACG	60.1	82
Ld_contig02690_PCRQ_R	GCGGTATTACCAAGACCAGTCAATC	59.8	
Ld_contig02530_PCRQ_F	GTGGGCAACAAGAAAGAGAAGAGAG	59.8	119
Ld_contig02530_PCRQ_R	CGGGAACCCTAAGGACGATATGG	59.8	
Ld_contig02668_PCRQ_F	AGGTGCTACCAAGTACAGTAGTTGAG	59.6	100
Ld_contig02668_PCRQ_R	GTTGCTCGTTTAAAGTTGATGCTGTG	60.4	
Ld_contig00126_PCRQ_F	GGTCTTCGCAAGCAAGGTATGTC	59.7	109
Ld_contig00126_PCRQ_R	GCTGGAGTATATGCTGTGTTTGTTC	59.8	
Ld_contig01413_PCRQ_F	GCGTGTAGTAAAGCTTGGGTTTC	59.7	108
Ld_contig01413_PCRQ_R	ATGTGGTGTAGTTGTTCATGCTTGC	60	
Ls_EF1a_F	TTTGCCTGCGGGCTCTCG	60.1	144
Ls_EF1a_R	TGGAGGGTAGCGAACGACATATC	59.5	
Ls_RPL36_F	CGTGAAGATGGTGAAGGAGGTC	59.8	125
Ls_RPL36_R	GCTTGGCGAACTTGTAGATGCG	60.2	
Ls_contig01122_F	AGCGGATGGATCAGGCAAAAGG	60	126
Ls_contig01122_R	GGCAACCGTTGTTGCACTG	60	
Ls_contig00733_F	GGCGAGGTTGTTAAGGACTGAG	59.1	109
Ls_contig00733_R	GGCACCACCGACTGCTCTG	59.9	
Ls_contig00239_F	CGGAGTCTGCCCTGCGGTAC	59.4	86
Ls_contig00239_R	TTCCGTATCCCTGACCGTGATG	59.6	
Ls_contig02470_F	AGGAAGTTGTGCGACGAGGTG	60.1	81
Ls_contig02470_R	TACGAGGTGTGCGGCAATAGC	60.3	
Ls_contig00467_F	CATCCGCCCGAGTATGAACAAAG	59.5	129
Ls_contig00467_R	GCCACAAATCCCGCATCCAAC	60.6	
Ls_contig01173_F	ACAGCACGACAATGGAGCAGAC	60.8	127
Ls_contig01173_R	TCATCCGCCCACTGATTATCC	60.3	
Ls_contig00344_F	CCAGCAGCACGACAGAAATG	59.4	91
Ls_contig00344_R	TTCTCATCGGCGGCTAACG	59.6	
Ls_contig00161_F	CGGGTTCGGCTTTGAGTGTAAAC	59.5	99
Ls_contig00161_R	CGTGGCGAAACCTTCCGTAAC	59.4	
Ls_contig00025_F	TGGAAGTTGCCGTTGTAGG	55.4	103
Ls_contig00025_R	GTCGTCAACATTAAACCTTGC	55.1	
Ls_contig02225_F	ACGCATAAATCGCCAATGTTG	55.5	66
Ls_contig02225_R	GGCAGACCACTGGAGACC	55.7	
Ls_contig01083_F	GCTCCGTGGGTTAGATGC	54.7	70
Ls_contig01083_R	GGCACGAACTAGGAACGG	55.1	
Ls_contig01374_F	GTCGTTCTTGGTCTGCTGG	55	111
Ls_contig01374_R	GATTATTCGTCCTTTCACAGC	55	
Ls_contig00134_F	CTCCTCAGACTTGTTCGTTATG	55	75
Ls_contig00134_R	AGCCTCTCACACTTACATCC	54.6	







**S3 Table.** List of ions identified in *L. spicata* and relative abundance peaks in control and grazed individuals at 6, 12, 24 and 48h of stress.

Ion	Control												Grazed																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											
	C16	C18	C20	C22	C24	C26	C28	C30	C32	C34	C36	C38	C40	C42	C44	C46	C48	C50	C52	C54	C56	C58	C60	C62	C64	C66	C68	C70	C72	C74	C76	C78	C80	C82	C84	C86	C88	C90	C92	C94	C96	C98	C100	C102	C104	C106	C108	C110	C112	C114	C116	C118	C120	C122	C124	C126	C128	C130	C132	C134	C136	C138	C140	C142	C144	C146	C148	C150	C152	C154	C156	C158	C160	C162	C164	C166	C168	C170	C172	C174	C176	C178	C180	C182	C184	C186	C188	C190	C192	C194	C196	C198	C200	C202	C204	C206	C208	C210	C212	C214	C216	C218	C220	C222	C224	C226	C228	C230	C232	C234	C236	C238	C240	C242	C244	C246	C248	C250	C252	C254	C256	C258	C260	C262	C264	C266	C268	C270	C272	C274	C276	C278	C280	C282	C284	C286	C288	C290	C292	C294	C296	C298	C300	C302	C304	C306	C308	C310	C312	C314	C316	C318	C320	C322	C324	C326	C328	C330	C332	C334	C336	C338	C340	C342	C344	C346	C348	C350	C352	C354	C356	C358	C360	C362	C364	C366	C368	C370	C372	C374	C376	C378	C380	C382	C384	C386	C388	C390	C392	C394	C396	C398	C400	C402	C404	C406	C408	C410	C412	C414	C416	C418	C420	C422	C424	C426	C428	C430	C432	C434	C436	C438	C440	C442	C444	C446	C448	C450	C452	C454	C456	C458	C460	C462	C464	C466	C468	C470	C472	C474	C476	C478	C480	C482	C484	C486	C488	C490	C492	C494	C496	C498	C500	C502	C504	C506	C508	C510	C512	C514	C516	C518	C520	C522	C524	C526	C528	C530	C532	C534	C536	C538	C540	C542	C544	C546	C548	C550	C552	C554	C556	C558	C560	C562	C564	C566	C568	C570	C572	C574	C576	C578	C580	C582	C584	C586	C588	C590	C592	C594	C596	C598	C600	C602	C604	C606	C608	C610	C612	C614	C616	C618	C620	C622	C624	C626	C628	C630	C632	C634	C636	C638	C640	C642	C644	C646	C648	C650	C652	C654	C656	C658	C660	C662	C664	C666	C668	C670	C672	C674	C676	C678	C680	C682	C684	C686	C688	C690	C692	C694	C696	C698	C700	C702	C704	C706	C708	C710	C712	C714	C716	C718	C720	C722	C724	C726	C728	C730	C732	C734	C736	C738	C740	C742	C744	C746	C748	C750	C752	C754	C756	C758	C760	C762	C764	C766	C768	C770	C772	C774	C776	C778	C780	C782	C784	C786	C788	C790	C792	C794	C796	C798	C800	C802	C804	C806	C808	C810	C812	C814	C816	C818	C820	C822	C824	C826	C828	C830	C832	C834	C836	C838	C840	C842	C844	C846	C848	C850	C852	C854	C856	C858	C860	C862	C864	C866	C868	C870	C872	C874	C876	C878	C880	C882	C884	C886	C888	C890	C892	C894	C896	C898	C900	C902	C904	C906	C908	C910	C912	C914	C916	C918	C920	C922	C924	C926	C928	C930	C932	C934	C936	C938	C940	C942	C944	C946	C948	C950	C952	C954	C956	C958	C960	C962	C964	C966	C968	C970	C972	C974	C976	C978	C980	C982	C984	C986	C988	C990	C992	C994	C996	C998	C1000	C1002	C1004	C1006	C1008	C1010	C1012	C1014	C1016	C1018	C1020	C1022	C1024	C1026	C1028	C1030	C1032	C1034	C1036	C1038	C1040	C1042	C1044	C1046	C1048	C1050	C1052	C1054	C1056	C1058	C1060	C1062	C1064	C1066	C1068	C1070	C1072	C1074	C1076	C1078	C1080	C1082	C1084	C1086	C1088	C1090	C1092	C1094	C1096	C1098	C1100	C1102	C1104	C1106	C1108	C1110	C1112	C1114	C1116	C1118	C1120	C1122	C1124	C1126	C1128	C1130	C1132	C1134	C1136	C1138	C1140	C1142	C1144	C1146	C1148	C1150	C1152	C1154	C1156	C1158	C1160	C1162	C1164	C1166	C1168	C1170	C1172	C1174	C1176	C1178	C1180	C1182	C1184	C1186	C1188	C1190	C1192	C1194	C1196	C1198	C1200	C1202	C1204	C1206	C1208	C1210	C1212	C1214	C1216	C1218	C1220	C1222	C1224	C1226	C1228	C1230	C1232	C1234	C1236	C1238	C1240	C1242	C1244	C1246	C1248	C1250	C1252	C1254	C1256	C1258	C1260	C1262	C1264	C1266	C1268	C1270	C1272	C1274	C1276	C1278	C1280	C1282	C1284	C1286	C1288	C1290	C1292	C1294	C1296	C1298	C1300	C1302	C1304	C1306	C1308	C1310	C1312	C1314	C1316	C1318	C1320	C1322	C1324	C1326	C1328	C1330	C1332	C1334	C1336	C1338	C1340	C1342	C1344	C1346	C1348	C1350	C1352	C1354	C1356	C1358	C1360	C1362	C1364	C1366	C1368	C1370	C1372	C1374	C1376	C1378	C1380	C1382	C1384	C1386	C1388	C1390	C1392	C1394	C1396	C1398	C1400	C1402	C1404	C1406	C1408	C1410	C1412	C1414	C1416	C1418	C1420	C1422	C1424	C1426	C1428	C1430	C1432	C1434	C1436	C1438	C1440	C1442	C1444	C1446	C1448	C1450	C1452	C1454	C1456	C1458	C1460	C1462	C1464	C1466	C1468	C1470	C1472	C1474	C1476	C1478	C1480	C1482	C1484	C1486	C1488	C1490	C1492	C1494	C1496	C1498	C1500	C1502	C1504	C1506	C1508	C1510	C1512	C1514	C1516	C1518	C1520	C1522	C1524	C1526	C1528	C1530	C1532	C1534	C1536	C1538	C1540	C1542	C1544	C1546	C1548	C1550	C1552	C1554	C1556	C1558	C1560	C1562	C1564	C1566	C1568	C1570	C1572	C1574	C1576	C1578	C1580	C1582	C1584	C1586	C1588	C1590	C1592	C1594	C1596	C1598	C1600	C1602	C1604	C1606	C1608	C1610	C1612	C1614	C1616	C1618	C1620	C1622	C1624	C1626	C1628	C1630	C1632	C1634	C1636	C1638	C1640	C1642	C1644	C1646	C1648	C1650	C1652	C1654	C1656	C1658	C1660	C1662	C1664	C1666	C1668	C1670	C1672	C1674	C1676	C1678	C1680	C1682	C1684	C1686	C1688	C1690	C1692	C1694	C1696	C1698	C1700	C1702	C1704	C1706	C1708	C1710	C1712	C1714	C1716	C1718	C1720	C1722	C1724	C1726	C1728	C1730	C1732	C1734	C1736	C1738	C1740	C1742	C1744	C1746	C1748	C1750	C1752	C1754	C1756	C1758	C1760	C1762	C1764	C1766	C1768	C1770	C1772	C1774	C1776	C1778	C1780	C1782	C1784	C1786	C1788	C1790	C1792	C1794	C1796	C1798	C1800	C1802	C1804	C1806	C1808	C1810	C1812	C1814	C1816	C1818	C1820	C1822	C1824	C1826	C1828	C1830	C1832	C1834	C1836	C1838	C1840	C1842	C1844	C1846	C1848	C1850	C1852	C1854	C1856	C1858	C1860	C1862	C1864	C1866	C1868	C1870	C1872	C1874	C1876	C1878	C1880	C1882	C1884	C1886	C1888	C1890	C1892	C1894	C1896	C1898	C1900	C1902	C1904	C1906	C1908	C1910	C1912	C1914	C1916	C1918	C1920	C1922	C1924	C1926	C1928	C1930	C1932	C1934	C1936	C1938	C1940	C1942	C1944	C1946	C1948	C1950	C1952	C1954	C1956	C1958	C1960	C1962	C1964	C1966	C1968	C1970	C1972	C1974	C1976	C1978	C1980	C1982	C1984	C1986	C1988	C1990	C1992	C1994	C1996	C1998	C2000	C2002	C2004	C2006	C2008	C2010	C2012	C2014	C2016	C2018	C2020	C2022	C2024	C2026	C2028	C2030	C2032	C2034	C2036	C2038	C2040	C2042	C2044	C2046	C2048	C2050	C2052	C2054	C2056	C2058	C2060	C2062	C2064	C2066	C2068	C2070	C2072	C2074	C2076	C2078	C2080	C2082	C2084	C2086	C2088	C2090	C2092	C2094	C2096	C2098	C2100	C2102	C2104	C2106	C2108	C2110	C2112	C2114	C2116	C2118	C2120	C2122	C2124	C2126	C2128	C2130	C2132	C2134	C2136	C2138	C2140	C2142	C2144	C2146	C2148	C2150	C2152	C2154	C2156	C2158	C2160	C2162	C2164	C2166	C2168	C2170	C2172	C2174	C2176	C2178	C2180	C2182	C2184	C2186	C2188	C2190	C2192	C2194	C2196	C2198	C2200	C2202	C2204	C2206	C2208	C2210	C2212	C2214	C2216	C2218	C2220	C2222	C2224	C2226	C2228	C2230	C2232	C2234	C2236	C2238	C2240	C2242	C2244	C2246	C2248	C2250	C2252	C2254	C2256	C2258	C2260	C2262	C2264	C2266	C2268	C2270	C2272	C2274	C2276	C2278	C2280	C2282	C2284	C2286	C2288	C2290	C2292	C2294	C2296	C2298	C2300	C2302	C2304	C2306	C2308	C2310	C2312	C2314	C2316	C2318	C2320	C2322	C2324	C2326	C2328	C2330	C2332	C2334	C2336	C2338	C2340	C2342	C2344	C2346	C2348	C2350	C2352	C2354	C2356	C2358	C2360	C2362	C2364	C2366	C2368	C2370	C2372	C2374	C2376	C2378	C2380	C2382	C2384	C2386	C2388	C2390	C2392	C2394	C2396	C2398	C2400	C2402	C2404	C2406	C2408	C2410	C2412	C2414	C2416	C2418	C2420	C2422	C2424	C2426	C2428	C2430	C2432	C2434	C2436	C2438	C2440	C2442	C2444	C2446	C2448	C2450	C2452	C2454	C2456	C2458	C2460	C2462	C2464	C2466	C2468	C2470	C2472	C2474	C2476	C2478	C2480	C2482	C2484	C2486	C2488	C2490	C2492	C2494	C2496	C2498	C2500	C2502	C2504	C2506	C2508	C2510	C2512	C2514	C2516	C2518	C2520	C2522	C2524	C2526	C2528	C2530	C2532	C2534	C2536	C2538	C2540	C2542	C2544	C2546	C2548	C2550	C2552	C2554	C2556	C2558	C2560	C2562	C2564	C2566	C2568	C2570	C2572	C2574	C2576	C2578	C2580	C2582	C2584	C2586	C2588	C2590	C2592	C2594	C2596	C2598	C2600	C2602	C2604	C2606	C2608	C2610	C2612	C2614	C2616	C2618	C2620	C2622	C2624	C2626	C2628	C2630	C2632	C2634	C2636	C2638	C2640	C2642	C2644	C2646	C2648	C2650	C2652	C2654	C2656	C2658	C2660	C2662	C2664	C2666	C2668	C2670	C2672	C2674	C2676	C2678	C2680	C2682	C2684	C2686	C2688	C2690	C2692	C2694	C2696	C2698	C2700	C2702	C2704	C2706	C2708	C2710	C2712	C2714	C2716	C2718	C2720	C2722	C2724	C2726	C2728	C2730	C2732	C2734	C2736	C2738	C2740	C2742	C2744	C2746	C2748	C2750	C2752	C2754	C2756	C2758	C2760	C2762	C2764	C2766	C2768	C2770	C2772	C2774	C2776	C2778	C2780	C2782	C2784	C2786	C2788	C2790	C2792	C2794	C2796	C2798	C2800	C2802	C2804	C2806	C2808	C2810	C2812	C2814	C2816	C2818	C2820	C2822	C2824	C2826	C2828	C2830	C2832	C2834	C2836	C2838	C2840	C2842	C2844	C2846





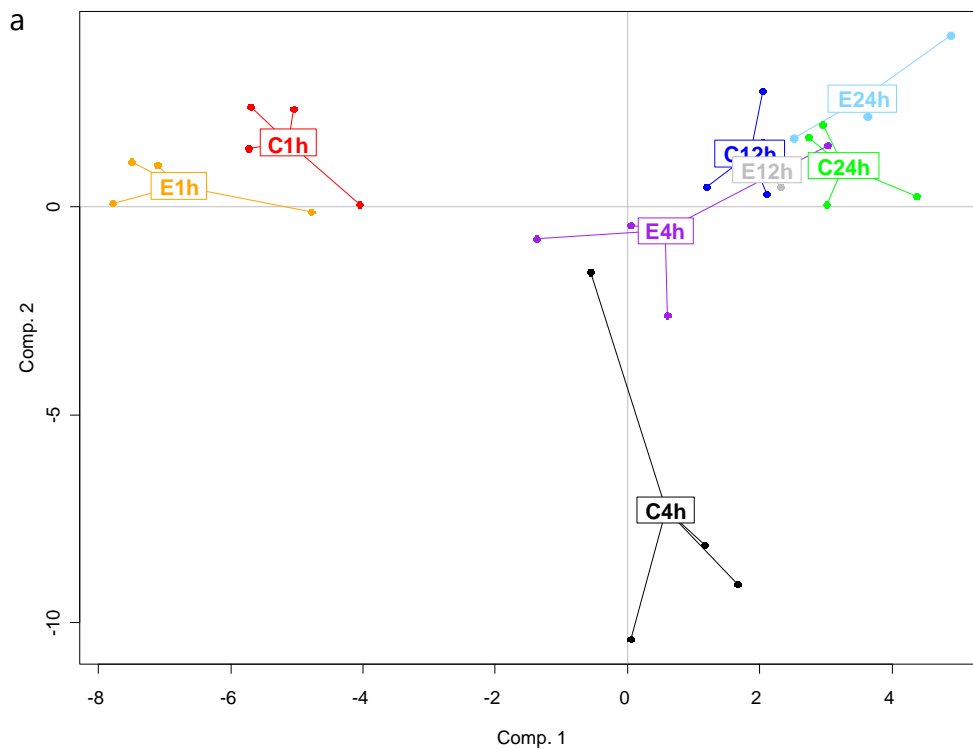


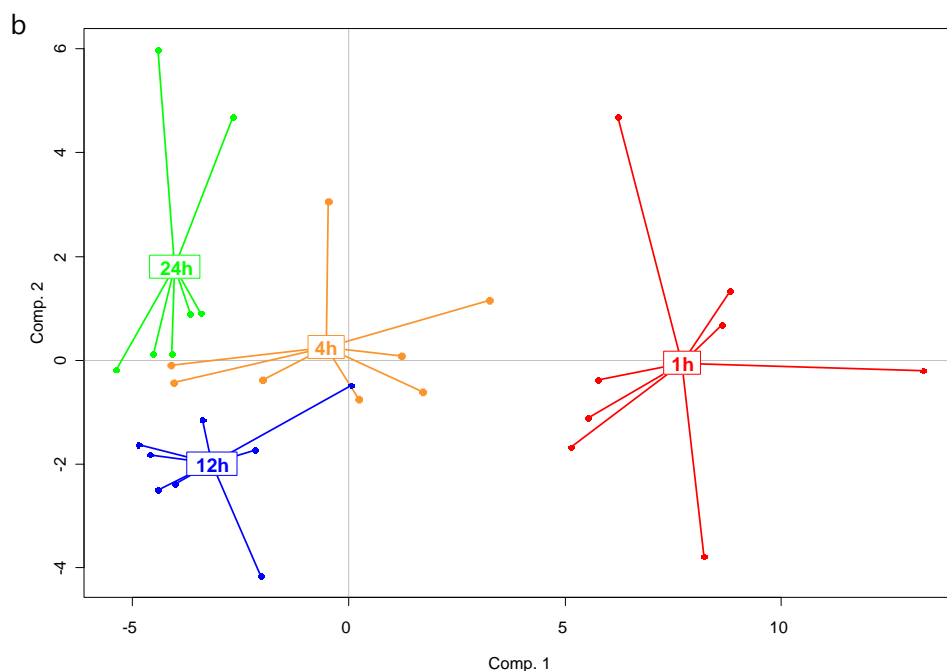




## II. Analysis of the exo-metabolome of an elicited alga

The surrounding seawater of batch cultures of elicited and control seaweeds was first analyzed by GC-MS from seawater samples collected at 1h, 4h, 12h and 24h after elicitation by GG. After data processing with Galaxy-W4M, the data matrix comprised a total of 1548 ions detected in samples, reduced to 296 selected ions after filtering. The results of the multivariate statistical analysis are represented in the PLS-DA in Fig 16a, which showed that, after 1h of elicitation, control and elicited algae seem to respond similarly, in contrast to elicited algae after 4h which seem to release different compounds from control algae. However, in samples collected later, after 12 and 24h of elicitation, control and elicited algae were not discriminated. The treatment (elicitation versus control) applied to the alga did not allow to discriminate their exo-metabolome (MVA.test, 999 permutations, NMC = 0.444, p-value = 0.317), whereas the sampling time significantly affected the bouquet of volatiles in the exo-metabolome of *L. digitata* (MVA.test, 999 permutations, NMC = 0.283, p-value = 0.001). Moreover, the sampling times were separated along the axis 1 (Fig 16b).

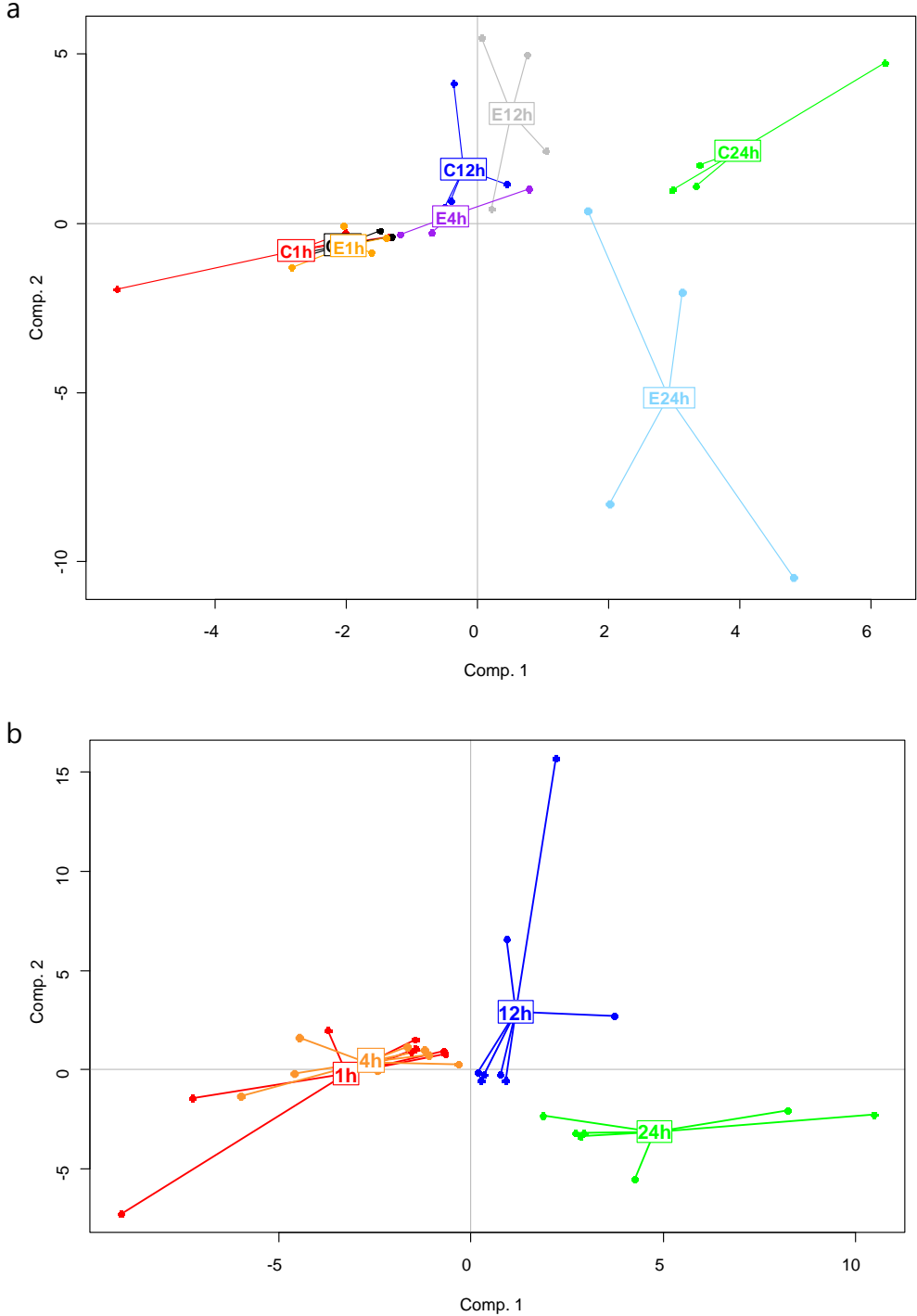




**Figure 16.** PLS-DA of the GC-MS analysis of the exo-metabolome of elicited (E) and control (C) *L. digitata* according to the sampling times (1h, 4h, 12h and 24h). Samples are represented after filtering of ions, finally obtaining 296 ions according to treatment and sampling time (a) and only according to sampling time (b).

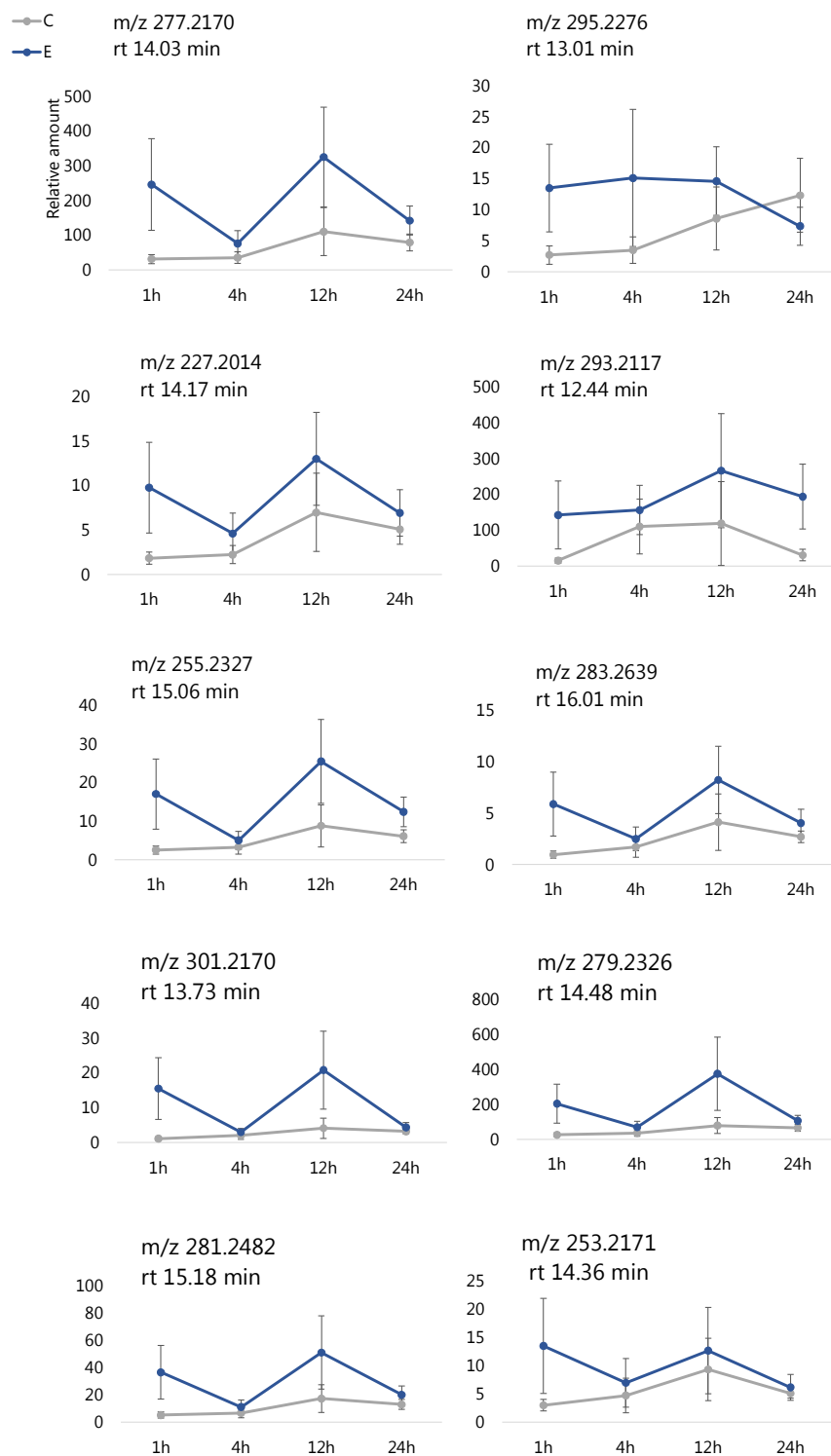
The surrounding seawater of batch cultures of elicited and control *L. digitata* after different incubation times (1h, 4h, 12h, 24h) was then analyzed in LC-MS in both negative and positive modes. After 3 trials of analysis in positive mode, the chromatograms still showed a contaminating element all along the running time, making them non interpretable. However, metabolomic analyses in LC-MS in negative mode resulted in a matrix of intensities of ion peaks detected in each sample. After processing with Workflow4metabolomics, 678 ions were detected and the final data matrix contained 457 ions after filtering of extreme running times and isotopes. These data were subjected to statistical analyses with R software, with the packages pls and RVAideMemoire, allowing to obtain Partial Least Squares – Discriminant Analysis (PLS-DA) and the Number of Misclassification (NMC) and the p-value associated. The PLS-DA in Fig 17a showed that if control and elicited algae sampled after 1h of elicitation did not seem to be separated, elicited algae after 4h seem to release different compounds from control algae. Like for GC-MS analyses, late sampling times (12h and 24h) could not allow to discriminate between control and elicitation treatments. When all sampling times were pooled, statistical analyses did not allow to conclude that the exo-metabolome was significantly different according to the treatment, either elicitation or control treatment (MVA.test, 999 permutations, NMC = 0.44688, p-value = 0.35). However, the PLS-DA showed in Fig 17b seems to discriminate the sampling times along axis 1, confirmed by multivariate statistical analyses which demonstrated that the composition of the exo-metabolome of an alga significantly depended on the sampling time in the experiment (MVA.test, 999 permutations, NMC = 0.34219, p-value = 0.001). Moreover, the number of misclassifications

(NMC) remained low, with less than 35% of variability which cannot be explained by the treatment and the sampling time.



**Figure 17.** Partial Least Squares - Discriminant Analysis (PLS-DA) of the LC-MS analysis in negative mode of the exo-metabolome of elicited (E) and control (C) *L. digitata* according to the sampling times (1h, 4h, 12h and 24h). Samples are represented after filtering of ions, finally obtaining 457 ions, according to treatment and sampling time (a) and only according to sampling time (b).

Some ions were selected as they were already identified as induced in response to biotic and abiotic stresses in brown algae, and mostly after copper stress in *L. digitata*. They could thus be annotated thanks to comparison of their m/z (mass/charge) ratio with the  $[M-H]^{-}$  ions of metabolomic databases, as putative fatty acids and their oxidized derivatives (Fig 18).



**Figure 18.** Relative amounts of some compounds annotated as fatty acids and their oxidized derivatives in surrounding seawater of elicited and control *L. digitata* plantlets, after 1h, 4h, 12h and 24h. The ions could be annotated as followed: 277.2170 m/z  $[M-H]^{-}$  rt 14.03 min:  $C_{18}H_{30}O_2$ , linolenic acid ; 295.2276 m/z  $[M-H]^{-}$  rt

13.01 min:  $C_{18}H_{32}O_3$ , hydroxyoctadecadienoic acid (HODE); 227.2014 m/z  $[M-H]^{-}$  rt 14.17 min:  $C_{14}H_{28}O_2$ , myristic acid; 293.2117 m/z  $[M-H]^{-}$  rt 12.44 min:  $C_{18}H_{30}O_3$ , HOTrE ; 255.2327 m/z  $[M-H]^{-}$  rt 15.06 min:  $C_{16}H_{32}O_2$ , palmitic acid; 283.2639 m/z  $[M-H]^{-}$  rt 16.01 min:  $C_{18}H_{36}O_2$ , stearic acid ; 301.2170 m/z  $[M-H]^{-}$  rt 13.73 min:  $C_{20}H_{30}O_2$ , eicosapentaenoic acid (EPA) ; 279.2326 m/z  $[M-H]^{-}$  rt 14.48 min:  $C_{18}H_{32}O_2$ , linoleic acid ; 281.2482 m/z  $[M-H]^{-}$  rt 15.18 min:  $C_{18}H_{34}O_2$ , oleic acid ; 253.2171 m/z  $[M-H]^{-}$  rt 14.36 min:  $C_{16}H_{30}O_2$ , palmitoleic acid. Grey lines correspond to the control treatment and blue lines to the elicitation treatment Mean relative amounts are represented  $\pm$  standard error. For each treatment at one sampling time, n=4. Statistical analyses performed between each pair of control and elicited algae at each sampling time did not show significant differences (Wilcoxon test,  $\alpha=5\%$ ).

Except for the 295.2276 m/z  $[M-H]^{-}$ , rt 13.01 min, the other annotated ions tended to be emitted 1h after elicitation, then decreasing after 4h, increasing again at 12h and finally decreasing at 24h. Control algae emitted less quantities of these compounds all along the experiment. Moreover, the relative amounts of 277.2170 m/z  $[M-H]^{-}$ , rt 14.03 min , 279.2326 m/z  $[M-H]^{-}$ , rt 14.48 min and 293.2117 m/z  $[M-H]^{-}$ , rt 12.44 min putatively corresponding to linolenic acid, linoleic acid and the oxylipin HOTrE respectively, were until 20 times more abundant than the other compounds. The 295.2276 m/z  $[M-H]^{-}$ , rt 13.01 min remained in high amounts during at least 12h, then decreasing at 24h. According to statistical analyses performed on each control/elicited pair sample, relative amounts of these compounds were not significantly different between the two treatments at each sampling time (Wilcoxon test,  $\alpha=0.05$ ).

## Discussion

### 1. A partial view of the exometabolome and putative signals emitted by an elicited alga

When metabolomic analyses were performed (3 years ago), the analytic technics that we used were not optimized for our samples. For example in GC-MS analysis, the sequence of sample injection did not comprise Quality Controls (QCs) which would have permitted a regular control of the quality of chromatograms and mass spectra all along the running time. Moreover, the tool Workflow4metabolomics is still under evolution, leading to different results obtained after metabolomic data processing between previous and current analyses. After LC-MS analysis of the exo-metabolome of an elicited alga, chromatograms obtained in the positive ionization mode presented an important contamination, making their interpretation not possible. This led to a lack of information on the composition of volatile compounds released in the medium. Thus, it should be interesting to make a new analysis in positive ionization mode, which would imply an optimization of the metabolomic analyses by LC-MS analysis, regarding the chemical gradient of elution. The results obtained in the positive mode would give supplementary information regarding, for instance, the content of the exo-metabolome in amino acids.

### 2. Changes over time of both endo- and exometabolome in response to multiple external factors

This work conducted on inducible chemical regulations in the kelps *L. digitata* and *L. spicata* in response to grazing allowed to get new information about the global changes of the endo- and the exo-metabolome of a grazed kelp, and about the response timing and the metabolic pathways that were activated upon attack. First, in contrast to analysis of the endo-metabolome which showed global different compositions in chemical compounds between grazed and ungrazed algae, the exo-metabolome was significantly different according to time. Results in both GC-MS analyses and in LC-MS in the negative mode allowed to discriminate samples collected early in time from those collected late, and the exo-metabolome seemed to be different between elicited and control algae after 4h of incubation. At late sampling times, the treatment effect could be hidden by the effect of the experimental design, which could for example cause a lack of oxygen or nutrients for the kelp from 12h of incubation. Ions that discriminate the two treatments could be hidden by other ions that are predominantly influenced by time. Moreover, there is often an important background noise in metabolomic analyses, which can hide the direct effects of a treatment on algal endo- and exo-metabolome.

### 3. A potential induction of the oxylipin pathway

Based on metabolic profiling data, both internal algal tissue and surrounding seawater analyses showed an accumulation and release of potential free fatty acids and oxylipins. After LC-MS analyses of the exo-metabolome of *L. digitata* after elicitation, 8 free fatty acids and 2 oxylipins could be annotated. Interestingly, the relative amounts of all the fatty acids were high 1h and 12h after GG elicitation, whereas one oxylipin remained in high amounts until 12h before decreasing at 24h. However, the exact nature of these compounds was not demonstrated. Complementary investigations should thus be performed in order to validate or refute the identification of the fatty acids and oxylipins annotated in LC-MS in negative mode. For that purpose, it would be essential to make metabolomic analyses of the samples in LC-MS/MS added up to analysis of chemical standards of these targeted compounds.

Thus, in addition to being accumulated in algal tissues, this complementary work highlighted that kelps also released some of these free fatty acids and their oxidized derivatives into the seawater in response to elicitation. These compounds were already described as emitted in the surrounding seawater after 3h and 8h of copper stress applied to *L. digitata* (Ritter et al., 2008; Ritter et al., 2014). Lab and field experiments on *L. digitata* had also shown that aldehydes, which are oxidized derivatives of polyunsaturated fatty acids (PUFAs), were emitted during and 1h after low tide, and after elicitation for 1h by oligogulonates (Goultquer et al., 2009). The chemical compounds that were emitted in the seawater after elicitation could thereafter participate to a potential signaling for a neighboring kelp. In the chapter 2, we explore this hypothesis and the potential responses of a “warned” neighboring kelp to grazing. Further studies need also to be done to determine the nature of the chemical compounds that could induce defense responses in the neighboring kelps.





## **Chapter II**

Distance signaling in kelps and defense against herbivores



## Introduction

Besides local responses inside algal tissues, kelps also respond to grazing stress through the release of volatile and soluble compounds into the surrounding seawater. Later on, these compounds can potentially be perceived by herbivores or by neighboring algae. In brown algal communities, distance signaling could play crucial roles mostly for prevention of a danger, such as during herbivore attacks. For example, waterborne cues emitted upon grazing were shown to induce higher concentrations of phlorotannins in *Ascophyllum nodosum* tissues which were less palatable than ungrazed algae (Toth and Pavia, 2000). In *Fucus vesiculosus*, distance signaling induced defense responses of the neighboring *Fucus* individuals (Rohde et al., 2004). These studies thus showed that waterborne signals emitted by the challenged alga can serve as warning signals to induce production of defense compounds inside algal tissues of neighbor conspecifics. They can induce metabolic modifications in the alga which perceives some of these signals. For example, in *F. vesiculosus*, secondary metabolites such as terpenoids, polyphenols and fatty acids are produced against herbivory, which also trigger gene regulation namely involved in lipid metabolism and in defense and stress responses (Flöthe et al., 2014). The kelp *L. digitata* was shown to release volatile polyunsaturated aldehydes and oxylipins, which are oxidized derivatives of fatty acids, in the medium upon biotic and abiotic stresses (Ritter et al., 2008; Goullitquer et al., 2009).

It seems that the efficiency of these chemical modifications in algal defense against herbivore attacks are both species- and grazer-specific. For instance, *Laminaria japonica* exposed to grazing by *Littorina brevicula* was less palatable for conspecific grazers during a subsequent grazing, while previous exposure of kelps to the abalone *Haliotis discus* did not decrease the palatability for a subsequent grazing by *H. discus* (Molis et al., 2008). Similarly, grazed *A. nodosum* by *L. obtusata* were less susceptible to further grazing than ungrazed algae, which was not the case after grazing by *Idotea granulosa* (Pavia and Toth, 2000). One likely explanation for these differences lies in the degree of specialization between the grazer and its host. When two species are intimately associated in an ecologically specialized relationship, as the result of a co-evolution between the two species, the defense mechanisms either are inefficient because the herbivore has adapted to avoid or tolerate these compounds, or display other functions in the biotic interaction. As opposed to the terrestrial environment, most marine herbivores are generalists (Hay and Steinberg, 1992), with only a handful marine species involved in specialized associations (Hay, 2009). In these cases, it seems that induced chemical cues produced in response to grazing have complex effects on specialized grazers, including their attraction instead of their repellence as also described in plants (Kessler and Baldwin, 2001). For instance, the location of food or hosts by grazers or pathogens can be facilitated by compounds like DMS (Dimethylsulfide) produced by the algae (Pohnert et al., 2007), leading to behavioral changes of the herbivore that increases its ability to find and consume algal food sources (Steinke et al., 2006). Coleman et al., (2007) found that feeding of *L. obtusata* on *A. nodosum* induced the attraction of two

predator species that could feed on the herbivore. Thus, specialist and generalist herbivores should interact differently with their algal host and, conversely, algal responses should be different according to the degree of specialization of the herbivore.

In the context of kelp/herbivore interactions, we hypothesized that a grazed kelp could have an impact, through chemical distance signaling, on metabolism regulations of neighboring algae, possibly leading to protection against grazers. Thus, in this chapter, we tested whether these chemical signals could have a role in algal defense against grazers through activation of metabolic pathways. Moreover, field observations on *L. spicata* revealed high amounts of *S. scurra* grazers on a single individual, while some neighbors could be free of grazers. We then hypothesized that grazing by herbivores could trigger metabolic modifications in grazed algal tissues and in the neighboring kelps, which could then modify herbivore behavior towards these kelps. Thus, we tested the effects of distance chemical signals on a subsequent grazing by specialist herbivores compared with a grazing by generalist herbivores. The chemical signals were generated either by elicitation with oligoguluronates as they were shown to activate innate immunity metabolism and priming effect (Küpper et al., 2002; Cosse et al., 2009; Thomas et al., 2011), or by a previous grazing, as we have shown in Chapter I that this induces metabolism changes after 48h. We hypothesized that the chemical signal is then transmitted by co-incubation between the sender and the receiving kelp individuals. Finally, the effects of these signals were assessed by monitoring the evolution over time of algal endo-metabolome, by measurement of the algal consumption by specialist and generalist herbivores, and by testing the modifications of herbivore behavior remotely.

The first part of the chapter is dedicated to the article which deals with ecological and chemical modifications in kelps upon grazing stress. The first purpose of this article was to determine the effects of chemical signals originating from a neighboring grazed kelp on herbivore behavior, through monitoring of algal consumption by grazers. Then, the effects of these chemical signals were investigated on algal metabolic regulations in *L. spicata* through metabolomic approaches. Then, the second part of this chapter aims at studying the effects of different algal grazing treatments on herbivore behavior by monitoring their attraction towards grazed or elicited kelps, or their receiving neighbors. In this part, not only specialist herbivores but also generalists were used to test their food preferences, through choice experiments for healthy kelps, elicited or grazed, co-incubated with a grazed or an elicited kelp, or using wounded stipes in the lab and in the field. First, elicited and healthy *L. digitata* were tested for preference of the generalist herbivore *Haliotis tuberculata* (abalones). Secondly, grazed *L. spicata* and co-incubated of grazed kelps were presented as food choices to the generalist herbivore *Tegula atra*. Then, attraction of *L. spicata*'s specialist herbivore *S. scurra* was tested for its preference for natural wounds on *L. spicata* stipes. Finally, a preliminary *in situ* experiment tested the attraction of *S. scurra* for artificial or natural wounds on *L. spicata*'s stipes.

# I. Ecological and chemical changes of *Lessonia spicata* to grazing by specialist herbivores

## General outline

In order to determine the effects of grazing stress on kelp defense responses, we performed non choice bioassays on *L. spicata* with its specialist herbivore *S. scurra* and metabolic profiling on the kelp submitted to different treatments. We tested first the effects of a direct grazing and a co-incubation with a grazed kelp on responses of *L. spicata* to a subsequent grazing. We compared the effects of a direct grazing treatment by *S. scurra* and a chemical elicitation treatment by oligoguluronates on algal consumption during a subsequent grazing by *S. scurra*. Results showed that Grazing but not Elicitation treatment influenced consumption by herbivores with a higher consumption of kelps that were previously grazed, than controls or elicited kelps. This difference was, however, significant only at the end of austral summer, but not during spring, suggesting a strong effect of seasonality on algal physiological response to grazing stress.

In order to investigate algal metabolic regulations in algae co-incubated with grazed kelps, algal metabolic profiling was performed after an indirect grazing of *L. spicata* by *S. scurra* compared to direct grazed kelps, according to time. Here we showed that the endo-metabolome of a grazed *L. spicata* was different from the one of its co-incubated neighbor, and from its respective control without any grazer. Moreover, a co-incubated kelp with a control alga without grazer showed a different metabolome from a co-incubated kelp with a grazed alga. Among these compounds, potential fatty acids and oxylipins could be found as induced after direct and indirect grazing treatments over the 2-day grazing. Thus, metabolite profiling analyses suggested that the kelp co-incubated with a grazed alga would perceive and integrate a soluble signal released into the seawater by the grazed alga. In the neighboring kelp, metabolic regulations seem to occur, especially regarding the fatty acid pathway and their oxidation.

Finally, this article allowed to conclude that responses of grazed kelp strongly depended on the season, in terms of weight gain and defense against grazers, in contrast to elicited kelps which were not subjected to these changes. In addition, algal consumption after Grazing treatments and metabolome analyses were in agreement. Direct grazing and co-incubation with a grazed kelp showed different grazing levels as well as different metabolomes. Therefore, direct grazing modified the metabolome of the kelp, then changing the one of its neighbor, and this could participate to defense reactions of the kelp.



## Article

# Ecological and chemical changes of *Lessonia spicata* to grazing by specialist herbivores

**Authors:** Cabioch L.<sup>1,2,3</sup>, Goulitquer S.<sup>1</sup>, Potin P.<sup>1</sup>, Leblanc C.<sup>1</sup>, Faugeron S.<sup>2,3</sup>

### **Affiliations:**

<sup>1</sup> Sorbonne Université, UPMC Univ Paris 06, CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France

<sup>2</sup> Sorbonne Université UPMC Univ Paris 06, Pontificia Universidad Católica de Chile, Universidad Austral de Chile, CNRS, UMI 3614 Evolutionary Biology and Ecology of Algae, Station Biologique de Roscoff, Roscoff, France

<sup>3</sup> Centro de Conservación Marina and CeBiB, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

Article in preparation



# Ecological and chemical changes of *Lessonia spicata* to grazing by specialist herbivores

Cabioch L.<sup>1,2,3</sup>, Goulitquer S.<sup>1</sup>, Potin P.<sup>1</sup>, Faugeron S.<sup>2,3</sup>, Leblanc C.<sup>1</sup>

<sup>1</sup> Sorbonne Université, UPMC Univ Paris 06, CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France

<sup>2</sup> Sorbonne Université UPMC Univ Paris 06, Pontificia Universidad Católica de Chile, Universidad Austral de Chile, CNRS, UMI 3614 Evolutionary Biology and Ecology of Algae, Station Biologique de Roscoff, Roscoff, France

<sup>3</sup>Centro de Conservación Marina and CeBiB, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

## Abstract

In response to grazing by herbivores, marine macroalgae have developed chemical defenses. A part of these chemical compounds are produced and kept within algal tissues, while others are emitted in the surrounding seawater, possibly constituting a distance signaling. Moreover, grazing by herbivores affects regulations of transcription in algae and modifications in metabolic pathways. In brown algal kelps, we have shown systemic responses in the brown alga *Laminaria digitata* upon defense elicitation and demonstrated a priming effect, induced by chemical cues present in seawater surrounding a kelp bed and/or released from neighboring brown algae. Such distant chemical signaling should have a major ecological role in structuring marine algal and herbivore communities. However, ecological impacts of this chemical signaling on kelp/herbivore interactions, as well as the nature of the chemical compounds produced in algal tissues remain unknown. Thus, in the context of kelp/herbivore interactions, we studied defense of *Lessonia spicata* against its specialist grazer *Scurria scurra*, present along Chilean coasts by means of bio-assays, and, simultaneously, we explored modifications of the algal metabolism by metabolomics approaches. We found that algal defense against grazers depends significantly on the season, especially between March and November. In March, *L. spicata* previously grazed were more eaten by grazers, thus less capable of defending themselves, compared with the end of the year. Moreover, metabolomics studies showed that the endo-metabolome of a grazed kelp was different from its neighbor, as well as from a naive kelp. These results should help to understand biochemical mechanisms in the ecological context of kelp defense against herbivores.

## Introduction

Kelp populations form complex ecosystems, including communities of micro- and macroscopic organisms living, reproducing and/or feeding in this environment. They are often subject to grazing by herbivores which live with them in the same ecosystem. Because they are sessile, kelps have to cope with these biotic stresses by setting up defenses against grazing by herbivores. It is now well known that chemical defense plays a crucial role in algae/herbivore interactions at different levels: directly and locally against grazers, systemically in the whole alga, and indirectly via interplant signaling. These chemical defenses may involve different classes of molecules. Among the best studies is the case of phlorotannins. Brown macroalgae, for instance, can defend directly against grazers by their specific ability to produce and store phlorotannins (Hay and Fenical, 1988). These defense chemical compounds were shown to be induced after grazing of *A. nodosum* by different *Littorina* species (Pavia and Toth, 2000) and *Idotea baltica* (Haavisto et al., 2010), then negatively affecting a subsequent grazing (Toth et al., 2007) by conspecifics and decreasing reproduction of grazers (Toth et al., 2005) or algal palatability (Haavisto et al., 2010). It was also found that algal defense responses were triggered by distance signaling via waterborne compounds. Indeed, after perception of waterborne cues by the alga *A. nodosum* induced after grazing by *I. baltica* or *Littorina obtusata*, the kelps contained higher concentrations of phlorotannins and were less palatable than control ungrazed algae (Toth and Pavia, 2000).

These studies show that waterborne signals emitted by the challenged alga can serve as warning signals to induce production of defense compounds inside algal tissues of neighbor conspecifics. Similar defense distance signaling was found in the brown alga *Fucus vesiculosus*, where grazing by *I. baltica* induced the release of a signal that triggered defense responses in neighboring *Fucus* individuals (Rohde et al., 2004) and in *A. nodosum* which uses waterborne signals for higher production of defense compounds (Toth and Pavia, 2000). Diatoms are also able to defend against grazing by copepods, by producing and emitting a wide range of polyunsaturated aldehydes, leading to a decrease of grazer reproduction (Pohnert, 2005). It is now well known that modification of the algal chemistry can change rapidly the susceptibility of algae to grazer consumption (Aguilera, 2011) and probably food preferences of herbivores, only a few hours after grazer consumption, and then disappear when grazing challenge ends (Aguilera, 2011).

Few studies so far have investigated the actual regulations of algal metabolism induced by distant signaling in response to grazer attacks. In *Fucus vesiculosus*, secondary metabolites such as terpenoids, polyphenols and fatty acids are produced against herbivory (Wahl et al., 2011). It was also shown to result in gene expression regulations, including up-regulation of genes involved in lipid metabolism, carbohydrate metabolism, intracellular trafficking, and defense and stress response (Flöthe et al., 2014). In *Laminaria digitata*, Küpper et al. (2001) first demonstrated that this brown alga recognizes and reacts

in response to application of cell wall polysaccharides from kelp, named as oligogulonates (GG). Another study showed that these chemical elicitors were able to trigger a higher protection of the kelp against further pathogen infections (Küpper et al., 2002). However, unlike bacterial lipopolysaccharides elicitors, GG elicitors are not able to induce release of fatty acids and accumulation of oxylipins in *L. digitata* (Küpper et al., 2006). Later, Goullitquer et al. (2009) found that, in response to different biotic and abiotic stresses, the kelp *L. digitata* releases volatile polyunsaturated aldehydes (PUAs) and oxylipins in the medium. These compounds may thus participate to a chemical signaling between kelps, as *L. digitata* was found to perceive waterborne cues present around chemically elicited neighboring plants, then changing early responses in metabolism as well as induction of genes (Thomas et al., 2011), also after 24-hour time elicitation by oligogulonates (Cosse et al., 2009).

It seems, however, that these chemical responses to grazing are grazer specific. Indeed, Pavia and Toth (2000) found that *A. nodosum* plants grazed by *L. obtusata* were less susceptible to further grazing than ungrazed algae, unlike when grazed by *I. granulosa* which had no effect on secondary attacks. Fresh *Laminaria japonica* exposed to *Littorina brevicula* were less palatable during a subsequent grazing than when previously consumed by the abalone *Haliotis discus* (Molis et al., 2008). One likely explanation for these differences lies in the specificity of the association between the grazer and its host. When two species are intimately associated in an ecologically specialized relationship, as the result of a co-evolution between the two species, the defense mechanisms either are inefficient because the herbivore has adapted to avoid or tolerate these compounds, or display other functions in the biotic interaction. For instance, Coleman et al. (2007) found that feeding of *L. obtusata* on *A. nodosum* induced the attraction of two predator species that could feed on the herbivore. Location of hosts by grazers or pathogens can be facilitated by compounds like DMS (Dimethylsulfide) produced by the algal host (Pohnert et al., 2007), leading to behavioral changes of the herbivore that increases its ability to find and consume algal food sources (Steinke et al., 2006).

In this study, chemical and ecological approaches were combined to investigate the nature of a chemical signaling between kelps in response to grazing by herbivores, and to test the effects of this signaling on metabolic regulations occurring into algal tissues and on herbivore deterrence. We used the kelp/herbivore couple *Lessonia spicata* and *Scurria scurra*, originated from Chile. *Lessonia spicata* (Suhr) Santelices (González et al., 2012) lives on the low intertidal of wave-exposed rocky shores of central and southern Chile where it is an ecologically and economically important resource (Vásquez, 2008). The kelp hosts a specialized herbivore *Scurria scurra* Lesson (Patellogastropoda, Gastropoda) that lives within its holdfast and at the basal part of the stipes (Santelices et al., 1980). *S. scurra* has a restricted range of host species: it can be found on the bull kelp *Durvillaea antarctica*, but this interaction is opportunistic as it depends on the relative abundance of the host. On the contrary, its association with *L. spicata* is independent of host relative abundance, indicative of its selectivity of this host species (Meynard, 2014). *S. scurra* lives and feeds in deep scars reaching stipe's medullary tissue, causing sever

deformation and depigmentation of the stipe. Only one limpet per scar and one scar per stipe are generally observed (Muñoz and Santelices, 1989). However, superficial grazing scars associated to *S. scurra* are frequently observed on upper parts of the stipes and on the fronds. Grazing is thus expected to trigger defense reactions from the kelp, in response to tissue damaging. This grazing effect on the host is, however, unclear. It is thought that this grazer could have a positive effect on *L. spicata* survival by pruning stipes, thus reducing drag force and preventing whole individual dislodgement by wave action (Santelices et al., 1980). Even if the costs and benefits of this biotic interaction have not yet been characterized, it is expected that the scars are sources of strong cellular stress and likely release different kinds of chemical compounds.

As kelp forests represent considerable food sources for marine herbivores, algae have to set up efficient chemical defenses. However, since *S. scurra* specifically feeds on *L. spicata*, it is expected to overcome algal defense compounds. The nature of these compounds and their role in distant signaling are unknown, as well as their actual effects on the herbivore's feeding behavior. Therefore, this study aimed at characterizing metabolic regulations in such grazed kelps, and assess whether these could lead to a better defense against subsequent grazing. Furthermore, the putative effect of distant signaling on neighbor individuals was investigated for both the metabolic changes and the grazing activity of *S. scurra*.

## **Materials and methods**

### **Algal and herbivore collections**

The kelp *Lessonia spicata* and its specialized herbivore *Scurria scurra* were sampled from rocky shores of Las Cruces (33°30'09"S : 71°37'59"O), Central Chile, between July 2014 and December 2015. Grazers were softly removed from basal parts of stipes during low tide, and kept cooled for transportation to the laboratory (no more than 1h). Then, they were fasted before the experiment, in a tank with constant seawater flow during 48 hours, at ambient temperature. Kelp harvesting consisted of collecting juvenile kelps. The whole *L. spicata* plantlets were sampled: one holdfast with all the stipes growing on it, with a maximum length of 20-50cm. Plantlets were stored in a tank with running seawater and air flow during one to two days for acclimation away from potential chemical signals of adult individuals in the natural stand.

### **General laboratory experimental set up**

The experiments were divided into two sequential phases: treatment phase and bio-assay by *S. scurra* grazing. For the treatment phase, the experimental unit consisted in two *L. spicata* plantlets placed in a 40 L-aquarium with constant seawater and air supplies. One of them was submitted to a stressful treatment, either chemical elicitation by oligogulonates or direct grazing by *S. scurra*, and

the other kelp was co-incubated with the treated one. In parallel, a control unit consisted in 2 plantlets, one being manipulated in the same way, but without any elicitor or grazer, and the other one co-incubated without any further manipulation. The co-incubation of the treated individual with its neighbor (or the respective controls) lasted 24 or 48 hours, according to the type of treatment (see below for details). For the bio-assay, one frond of each kelp (for the treated kelps, the frond exposed to the stressful treatment) was cut at the base of the frond, and then placed individually in separate plastic boxes of 6L with constant seawater flow for four days, together with two *S. scurra* grazers put on the frond. Each frond was blotted on paper towel to remove liquid water from surface and weighted before the bio-assay and after two and four days of grazing by *S. scurra*. In addition, the length and width of all grazers used for the bio-assay were measured, to calculate algal consumption standardized by grazers' size. Ten replicates of this experimental unit were set for each treatment, i.e. grazing or elicitation.

As the grazing assay was based on algal consumption, we have analyzed the effects of the experimental set-up and sampling time on algal weight change, to account for non-feeding-related changes in wet mass (i.e. autogenic changes). In the treatment phase of the experiment, two kelps were placed in an aquarium and treated following the same protocol (Grazing and Elicitation). Fronds were then cut, blotted and weighted, and put separately in 6L-boxes without any grazers. These fronds were then weighted after two and four days in seawater. Each experiment was independently run three times for each type of treatment.

### **Grazing experiment**

Two individuals of *S. scurra* were first deposited on one frond of 10 *L. spicata* plantlets kept in a net, to avoid herbivore escaping. Kelps in nets were named DG (direct grazed), whereas the other was named IG (indirect grazed, e.g. not directly in contact with the grazer, but co-incubated with the DG alga). The controls used for each treatment were named C<sub>DG</sub> (Control direct grazed) and C<sub>IG</sub> (Control indirect grazed) respectively. After 48 hours of co-incubation, the most grazed frond of DG and one frond of IG, C<sub>DG</sub> and C<sub>IG</sub> plantlets were selected for the bio-assay.

### **Elicitation experiment**

For chemical elicitation, alginate oligoguluronates (poly-alpha-1,4-L-guluronic acid blocks, GG blocks) were prepared by acid hydrolysis according to Haug et al. (1974), using sodium alginate from *Laminaria digitata* stipes (Danisco, Landerneau, France). The homopolymeric blocks of GG were used as an elicitor at a final concentration of 150 µg.mL<sup>-1</sup> as described in Küpper et al. (2001). Elicitation was done by placing, during 1 hour, one frond of the directly elicited individual (E) in a 50 mL-Falcon tube filled with 150 µg.mL<sup>-1</sup> GG dissolved in seawater. The Falcon tube was adjacent to the aquarium so the integrity of the individual was kept, and no more than 5 cm of frond tissue was exposed to air, between the aquarium and the tube. The control of Elicitation treatment was processed similarly, but the

Falcon tube was filled with seawater only. These treated fronds (E and C<sub>E</sub>) were then rinsed with seawater and replaced in the aquarium. In August, seawater was renewed 1 hour after elicitation and let in the aquarium for 24 hours. In November, plantlets were placed in aquarium with constant water supply. Fronds elicited in GG solution (E) or immersed in seawater (C<sub>E</sub>), and fronds from the co-incubated kelps (IE and C<sub>IE</sub> respectively for Indirectly Elicited and Control indirectly elicited) were cut at the stipe, blotted and weighted for the bio-assay.

### **Metabolite profiling and data analysis**

The kinetics of metabolism regulations associated with kelp responses to herbivory for directly grazed and co-incubated individuals was analyzed from separate experiments, performed in April 2010. Grazing by *S. scurra* was permitted during 6, 12, 24 and 48 hours, and two fragments per kelp were then separately frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  for analysis. In parallel, similar set up was applied to control plantlets. Three replicates were taken per condition at each sampling time.

For each treatment (C<sub>DG</sub>, DG, C<sub>IG</sub>, IG) and sampling time (6, 12, 24 and 48h), metabolic extracts were obtained from 100 mg of frozen algal powder with 1 mL MeOH:H<sub>2</sub>O (8:2), as described in Ritter et al. (2014). In each sample,  $1.25\ \mu\text{g}\cdot\text{mL}^{-1}$  12-OH-lauric acid was added as an internal standard for the negative mode analysis. Aliquots of 50  $\mu\text{L}$  of each extract were used for metabolomic analyses. They were first separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an Electro Spray Ionization (ESI) source running on the negative ionization mode, as described in Ritter et al. (2014). Metabolic samples were analyzed using a 1.9  $\mu\text{m}$  Thermo Hypersil Gold C18 column (100 x 2.1 mm) and the mobile phase (0.1% acetic acid in water–acetonitrile) was programmed from 100 : 0 to 5 : 95 acetonitrile–water (v : v). Xcalibur 2.1 software was used for instrument control and data acquisition.

Mass spectra data were processed by XCMS on the online version (Galaxy-Workflow4metabolomics, Giacomoni et al., 2014), after conversion of raw spectra to MzXML format. Data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples necessary at 0.7. For correction of retention time, the obiwrap method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 10. Fillpeaks step was used with the chrom filling method. Finally, annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. The final table contained ions abundances in samples, which corresponded to peak areas.

Data given by CAMERA contained 678 ions. Normalization was done first by dividing integrated areas by the highest one of the same sample, and then by dividing the areas of one ion by the highest in

all the samples. This would allow to compare the relative amount of one ion in different samples. After manual elimination of background and redundant (isotope) ions, the final table of data contained 514 ions.

### **Statistical analyses**

The algal weight gain was evaluated according to the season and to the treatment applied to the kelp using 1-way ANOVAs followed by Student-Newman-Keuls (SNK) post-hoc tests for comparisons of the mean weight gain. Algal consumption by herbivores during bio-assays after Grazing treatment was analyzed using Kruskal-Wallis test for comparison of mean consumption between seasons when normal distribution was not respected, or 2-ways ANOVAs and SNK tests to compare the mean consumption according to season and treatment, when appropriate. For the Elicitation experiment, algal consumption variations were considered separately in March and in November. Data were then analyzed using 1-way ANOVAs followed by SNK tests for comparisons of mean consumption depending on the Elicitation treatment. Statistical analyses of data were carried out using R.

Metabolomic analyses were performed using Partial Least Squares - Discriminant Analysis (PLS-DA), followed by extraction of the most discriminant ions. Where possible, annotation of ions was made from previous studies (Ritter et al., 2008). The free softwares R and SIMCA were used to perform statistical analyses.

## **Results**

### **Algal weight gain after Grazing treatment**

The lab experiments were conducted in different periods of the year. Figure 1A shows the percentage of weight gain of *L. spicata* in March and in November, in all samples from both Elicitation and Grazing treatments. It shows that, after a Grazing treatment (for DG and IG as well as for C<sub>DG</sub> and C<sub>IG</sub> treatments), the rate of weight gain is significantly higher in November than in March (F=4.46, p=0.04). Weight increase was higher in November than in March after the first two days following Grazing treatment, and this difference remained constant during at least 2 days of the experiment (F=4.3478, p=0.042). During this November experiment, weight gain was significantly higher for C<sub>IG</sub> treated kelps compared to the other treated fronds after two days (F=5.90, p=0.01) as well as after four days (data not shown, 1-way ANOVA; F=3.70, p=0.04). However, for the Elicitation treatment, there was no significant difference of algal weight gain between March and November, after two days (Fig 1A; F=0.13, p=0.72) as well as after four days (F= 1.03, p=0.32).

### **Algal consumption after Grazing treatment**

The real consumption of algae by grazers was evaluated by weighting kelps after two days and four days of bio-assay corrected by the specific algal weight gain of the corresponding season, according

to the treatment (Grazing or Elicitation). Algal consumptions were corrected using the formula:  $M2 - r * M2 / 100$  in which M2 is the weighted mass of the alga after 2 or 4 days of bio-assay, and r is the rate of weight gain of the frond.  $r = 0.9\%$  in March and  $1.7\%$  in December after two days of bio-assay;  $r = 2.1\%$  in March and  $3.5\%$  in December after four days.

Whatever the treatment applied to the kelps and the season, there was no significant differences in *S. scurra* consumption rate during the first 2 days of grazing (2-ways ANOVA, see Table 1A). However, after four days of bio-assay, DG treated fronds lost higher amounts of biomass due to grazing compared to any other treatment (Fig 2), and this was statistically significant in March only (2-ways ANOVA SNK test, see Table 1B). There was no significant differences in consumption rate of the co-incubated fronds. There was a significant effect of season, with a higher biomass loss in March than in December.

### **Elicitation treatment has no effect on algal consumption**

Season did not have a significant effect on algal weight gain after Elicitation treatment (Fig 1). Therefore, frond weights were corrected by  $r = 2.1\%$  after two days and  $r = 2.9\%$  after four days. There was higher herbivore consumption during the August experiment than in November, however the experiment was made in slightly different ways in August and in November. Therefore, this comparison between seasons must be considered with caution. In both seasons, no effect of elicitation, either direct (E) or indirect (IE), was detected on herbivore consumption rate. The same pattern was observed after two days of bio-assay (Fig 3), for both March (1-way ANOVA;  $F=0.28$ ,  $p=0.84$ ) as well as November (1-way ANOVA;  $F=0.49$ ,  $p=0.69$ ), and after 4 days of bio-assay (Fig S1), ( $F=0.37$ ,  $p=0.77$  in March;  $F=0.81$ ,  $p=0.50$  in November).

### **Metabolic changes after direct and indirect grazing**

Global PLS-DA, including data of 514 selected ions from all the samples analyzed in negative ionization mode, did not discriminate any Grazing treatment at any sampling time (Fig 4A). However, pairwise comparison showed that grazed kelps (DG) were clearly discriminated from controls without grazer ( $C_{DG}$ ; Fig 4B). The same pattern was observed when comparing indirect-grazed kelps (IG) with the respective controls ( $C_{IG}$ ; Fig 4C), as well as when comparing co-incubated algae (IG) with directly grazed (DG; Fig 4D), evidencing that different metabolic regulations occurred after the different Grazing treatments.

When focusing on the kinetics, some ions discriminated the four Grazing treatments, in particular compounds like fatty acids and oxylipins, known to be generated in response to an oxidative stress (Ritter et al., 2014). Their relative amounts (obtained after normalization) are represented in Figure 5 in the different Grazing-treated kelps, during the four sampling times. Eicosapentaenoic acid (EPA) was mostly induced in grazed kelps from 24h of grazing, and its relative amount increases until at least 48h



compared to control kelps. The oxylipin 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) was mainly produced in C<sub>DG</sub> and DG kelps after 6h of grazing. After 24h, IG kelps produced the highest amount of 13-HODE, two times more than its control C<sub>IG</sub>. At 48h, the amount of this compound decreased in IG kelps, more than three times less than in the DG and C<sub>IG</sub> kelps. Two phytoprostanes (Fig 5 and S2) were also produced in higher amount in the DG kelps after 6h of grazing, and one of maintained a constant relative amount from 12 to 48h of grazing, while the other dropped at 48h. On the contrary, IG kelps had a lower induction of one phytoprostane (Fig 5) at 6h of grazing, but then reached higher relative amount as in DG kelps, and finally decreased at 48h. Co-incubation with control kelps also induced production of this phytoprostane after 12h. Prostaglandin J (Fig 5) was induced in all Grazing-treated kelps after 6h, and then decreased after 24h, except in IG kelps at this sampling time. The same pattern was observed for prostaglandin B (Fig S2), except that its relative amount remained high until 24h in DG kelps.

## **Discussion**

### **Seasonality of algal weight gain**

Besides grazing stress, *Lessonia spicata* lives in a highly stressful environment, the low intertidal, submitted to strong variation in temperature, brightness or salinity, which influence its physiology as well as its metabolism. Here we demonstrate that season has a significant effect on algal weight gain after a Grazing treatment. In central and northern Chile, coastal environments are under the influence of upwelling events, which are stronger during austral spring and early summer (i.e. between September and January; Vasquez et al., 1998; Blanco et al., 2001; Vega et al., 2005). Upwelling brings a massive supply of nutrients and cools down seawater's temperature nearshore, strongly stimulating vegetative growth in kelps (Vásquez et al., 1998).

In November, the increase in weight was higher for C<sub>IG</sub> kelps than any other Grazing-treated kelps, which can be interpreted as the consequence of a lower stress induced by the treatment but also the experimental manipulation. Indeed, this was the least manipulated treatment, with no influence of co-incubation of a treated kelp. DG and C<sub>DG</sub> were comparable in that both were placed in nets, and showed very similar weight gain. On the contrary, IG kelps, co-incubated with DG individuals, had the lowest registered weight gain (although the average value did not differ statistically from the remaining treatments). This result suggests some kind of effect of the hypothesized distant chemical signaling produced by DG kelps.

Interestingly, these differences were not observed during Elicitation experiments. First, the absence of differences among treatments of algal weight gain suggests that Elicitation did not cause any strong physiological effect on the kelps. Second, algal weight gain remained constant during upwelling and non-upwelling seasons for Elicitation experiments, as opposed to Grazing experiments. This strongly suggests that Grazing did cause some stress, and that this stress had more negative effects on

growth rate during the non-upwelling season. The great similarity of weight gain between November Grazing experiments and Elicitation experiments (any season) might be interpreted as a higher amount of resources during the upwelling season, allowing grazed kelps (either directly or indirectly through co-incubation) to cope with that level of stress, and maintain their growth rate.

### **Algal consumption after direct grazing or elicitation**

We also showed that algal consumption by *S. scurra* varied according to the different Grazing treatments but not according to Elicitation treatments. This was already observed in the brown alga *Ascophyllum nodosum*, where mechanical damages showed no induction of defenses against herbivores, nor significant changes in phlorotannin levels in algae, compared with grazing by *Littorina obtusata* (Pavia and Toth, 2000). In *Fucus vesiculosus*, direct feeding of *I. baltica* and *L. littorea* induced chemical defence in *F. vesiculosus*, while simulated herbivory (i.e. mechanical damage) did not trigger algal defense (Rohde et al., 2004). Similarly, Coleman et al. (2007), in a choice experiment, demonstrated that artificially damaged *A. nodosum* and naïve fronds were equally attractive to the grazer *Lipophrys pholis*, while grazed algae were clearly more attractive than artificially damaged ones. Thus, it seems that algae are able to discriminate between artificial damage and grazing, and induce specific responses that modify the behavior of the grazer. Our experiments show that *L. spicata* also has this capacity, and that chemical elicitation by oligosaccharides is not an artificial substitute to elicitors of responses to natural grazing by herbivores.

Grazing induced changes in herbivore consumption rate: DG fronds were significantly more consumed than other Grazing-treated fronds after 4 days of bio-assay. This pattern seems contrary to what is expected under the establishment of defense mechanisms. There are actually several examples of positive feedback of grazing rate. Herbivore-induced *A. nodosum* were significantly more attractive to *L. pholis* grazers (Coleman et al., 2007). At least two mechanisms could be hypothesized to explain this pattern. One is that the kelp is not able to cope with the stress caused by the grazing, leading to uncontrolled molecular responses during the oxidative burst. Such kind of response has been evidenced for some abiotic stresses, like heavy metal contamination. For instance, oxidative stress caused by copper contamination in *L. berteroana*, a sister species of *L. spicata*, causes an uncontrolled lipoperoxide accumulation, leading to cell damage and dysfunction (Contreras et al., 2009). Such cellular deregulation is likely limiting the seaweed's capacity to efficiently activate defense mechanisms, providing an opportunity for the herbivores to consume unprotected tissues. An alternative hypothesis is that the herbivores are actually able to sense the chemical compounds released by the algae during grazing, inducing some kind of behavioral change. Our experiments were conducted with *S. scurra* which is a specialist herbivore selectively using *L. spicata* for shelter and food (Meynard, 2014). The behavior of this herbivore includes digging hole on stipes and holdfasts, and even though the limpets are able to move along stipes and fronds, they always go back to holes during low tide. Therefore, they

probably have the capacity to locate the holes, which are grazed tissues, by sensing the chemical cues produced by the holes. Moreover, because specialist herbivores are expected to have the capacity to overwhelm the repellent effect of defense compounds, the increase in consumption rate might actually be a strategy to compensate the loss of food quality. Pavia and Toth (2000) found that phlorotannin levels inside *A. nodosum* tissues increased only after grazing by the fucoid specialist herbivore *L. obtusata*, but not after grazing by a generalist grazer. In this case, increase in toxic compounds in the tissues is associated with an increase in consumption rate, probably as a compensatory process. Therefore, the increase in consumption rate of *S. scurra* is not indicative of a failure of defense mechanisms. Rather, it shows that the herbivore senses the state of the algae, and modifies his feeding behavior accordingly.

Interestingly, the increase in consumption rate was statistically significant only in March, which is when upwelling is weaker. We have shown that growth rate is reduced in March. Altogether, this could suggest that *L. spicata* is less able to produce defense compounds against grazers than in upwelling periods. This is in agreement with the study of Molis et al. (2006) which shows that season has an effect on induced defenses in *Ecklonia* against *Littorina* after direct grazing or co-incubation with a grazed kelp. The study made by Rohde and Wahl (2008) showed that grazing by *Idotea* follows a seasonal pattern, with a peak of grazing in summer, then decreasing in early winter. Seasonal variations in chemical composition of kelps could also explain changes of responses against herbivores depending on the period of the year (Schiener et al., 2014). Such hypothesis of seasonal regulation of defense mechanisms would however require further experimental testing to confirm this pattern on *L. spicata*.

### **Consumption of co-incubated *L. spicata* with grazed or chemically elicited algae**

Grazing was not significantly affected by co-incubation with treated (e.g. grazing or elicitation treatment; IG and IE) *L. spicata*. However, the lowest registered values of grazing were observed in kelps co-incubated with DG individuals in November's Grazing experiment. This tendency seems to support the hypothesis that priming exists and increases the protection of the kelps against grazing. Thomas et al. (2011) showed that the kelp *Laminaria digitata* was able to induce more rapidly specific gene regulations when co-incubated with elicited algae, and that they had a different pattern of oxidative burst and volatile emissions. More recently, distant signaling among *A. nodosum* was evidenced with the herbivore *L. obtusata*, which had a lower consumption rate on primed seaweeds (Flöthe and Molis, 2013). Rohde et al. (2004) demonstrated that grazing by *I. baltica* on *F. vesiculosus* released water-borne cues that triggered induced defenses in neighboring algae, as previously found in *A. nodosum* in response to grazing by *L. obtusata* (Toth and Pavia, 2000).

The fact that we observe a tendency of decreased grazing in IG fronds only in November further reinforces the idea that upwelling, by allowing a higher primary productivity, could also improve protection against different stresses, including grazing.

### **Metabolic profiling of Grazing-treated *L. spicata***

Grazing differences observed in bio-assays after a Grazing treatment can be associated with changes in algal endo-metabolome. Kelps previously exposed to a direct grazing had a different global metabolome than their respective control, as well as when compared to co-incubated kelps. Actually, the observation of three different metabolic profiles matches the observation of 3 groups of treatments affecting grazing rates (i.e. increase rate in DG, decreased rate in IG, as compared to all the remaining treatments and controls). Changes in metabolite profiles were also showed in the red alga *Gracilaria vermiculophylla* after grazing (Nylund et al., 2011; Rempt et al., 2012) and mechanical wounding (Nylund et al., 2011), with a high increase of prostaglandins compared with the species *G. chilensis* (Rempt et al., 2012). In red algae, Bouarab et al. (2004) found out some chemical compounds produced during challenging by defense elicitation, like polyunsaturated fatty acids, prostaglandins as well as oxylipins which confer higher resistance to the alga when challenged by a green algal endophyte. Moreover, Ritter et al. (2008) found that prostaglandins were produced after 8 hours of exposure to copper in *L. digitata*, as well as high concentrations of 13-HODE, and a wide range of EPA derivatives upon copper stress. 13-HODE was also found in the kelp *L. digitata* after challenge with lipopolysaccharides (LPS) from bacteria (Küpper et al., 2006) and in the red alga *Chondrus crispus* after exposure to *A. operculata* elicitors (Bouarab et al., 2004). EPA was already shown to be retained in damaged cells (Leflaive and Ten-Hage, 2009) and linked to polyunsaturated aldehydes (PUAs) released in the medium via lipoxygenases (Fontana et al., 2007) to prevent further attack around the organisms. In previous studies, EPA has been shown to be released in high amounts by diatoms upon cell damage, and highly toxic for crustacean herbivores (Jüttner, 2005). Galloway et al. (2012) and van Ginneken et al. (2011) showed that brown and red algae shared a basic common abundance of EPA, including *Laminaria hyperborea*. More recently, phytoprostanes were found in a wide variety of macroalgae (Barbosa et al., 2015). Other compounds like phlorotannins were also identified in brown algae (Toth and Pavia, 2000) in response to grazing, but it was suggested that they were not the sole compounds responsible for the anti-herbivory activity.

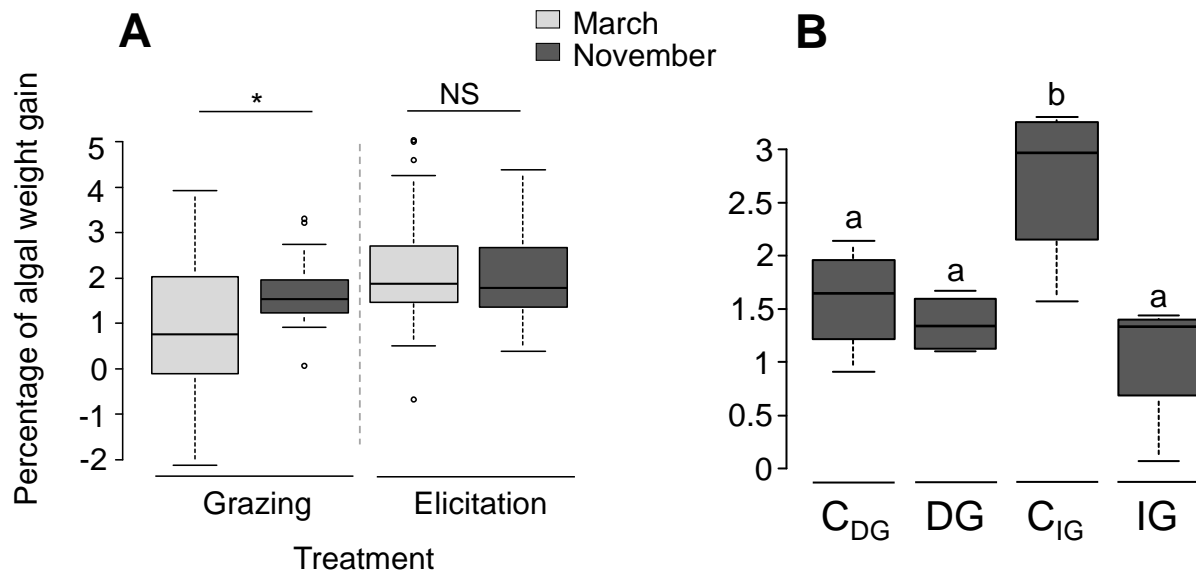
## **Conclusion**

Thus, algal defenses in response to herbivores depends on the period of the year. Indeed, during upwelling season in Chile (September - January), when nutrients and light are widely available for kelps, growth and weight gain of kelps are more important than in the rest of the year. Kelps may then defend more efficiently against herbivores in this season. On the contrary, non-upwelling season conditions would not allow previously grazed kelps to defend efficiently, leading to weakening of the kelp and invasion of *S. scurra* herbivores on one *L. spicata* plant. In upwelling season, these directly grazed kelps could warn their neighbors via waterborne cues, which might decrease consumption of these neighbors during a subsequent grazing. However, these mechanisms do not seem to be occurring under chemical Elicitation. Indeed, whatever the season, direct and indirect Elicitation do not seem to modify algal weight gain, nor defense responses against grazers. This might suggest that the kelp is able to discriminate between different kinds of tissue damage by activating different metabolic pathways, and that the herbivore is sensitive to the specific metabolic status of the kelp.

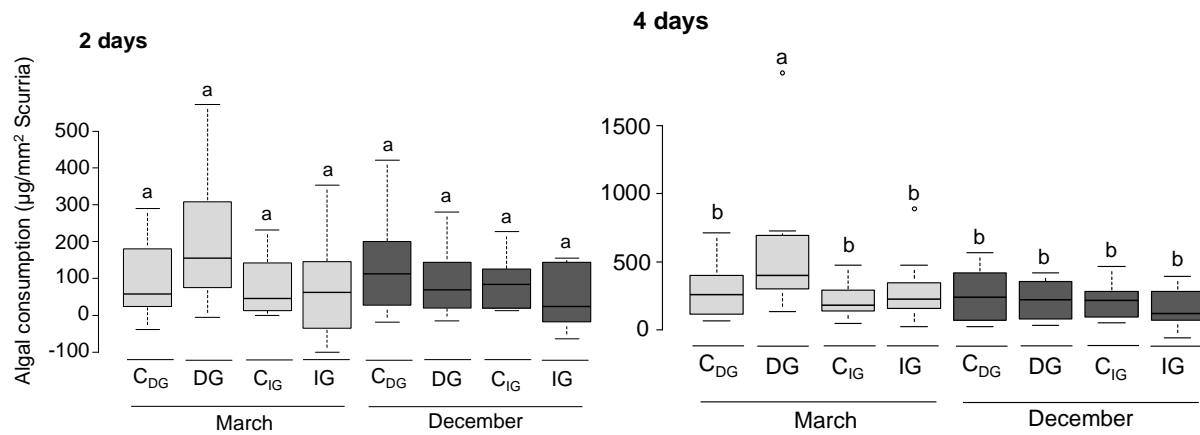
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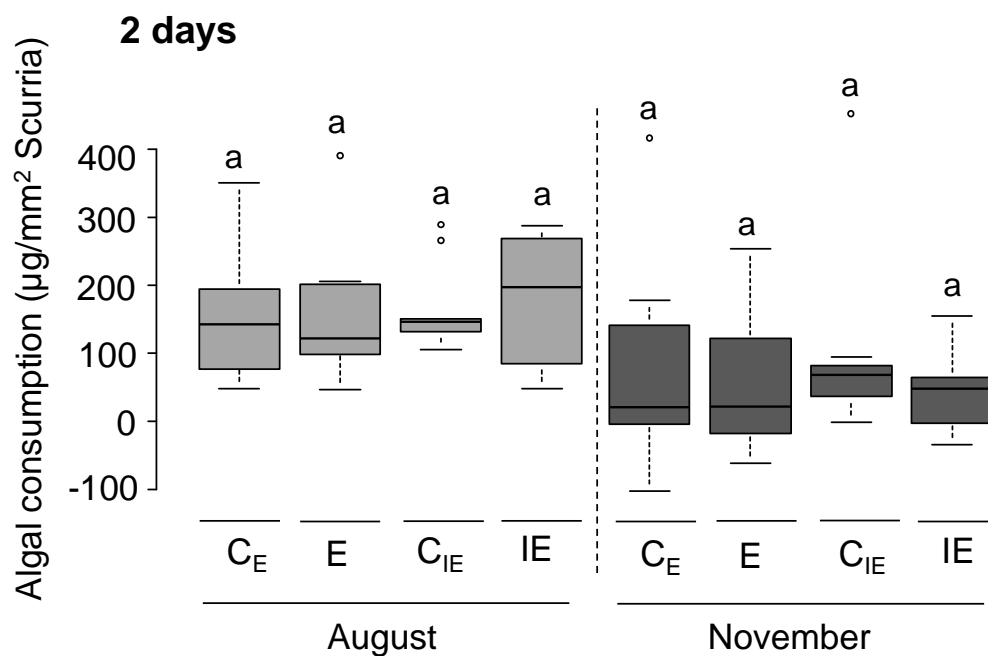
## Figure legends



**Fig. 1.** Percentage of weight gain of *L. spicata* A) Weight gain in March and in November, following Grazing or Elicitation treatment. Significance of mean difference is indicated: \* $p < 0.05$ , NS =  $p > 0.05$ , as determined from 1-way ANOVA tests. Grazing experiment,  $n=40$  in March,  $n=16$  in November; Elicitation experiment,  $n=36$  in March,  $n=15$  in November. B) Weight gain of kelps exposed to the different Grazing treatments C<sub>DG</sub>, DG, C<sub>IG</sub>, IG in November;  $n=4$  for each treatment. Letters shared in common between the groups indicate no significant difference (ANOVA,  $p < 0.05$ ; SNK test).

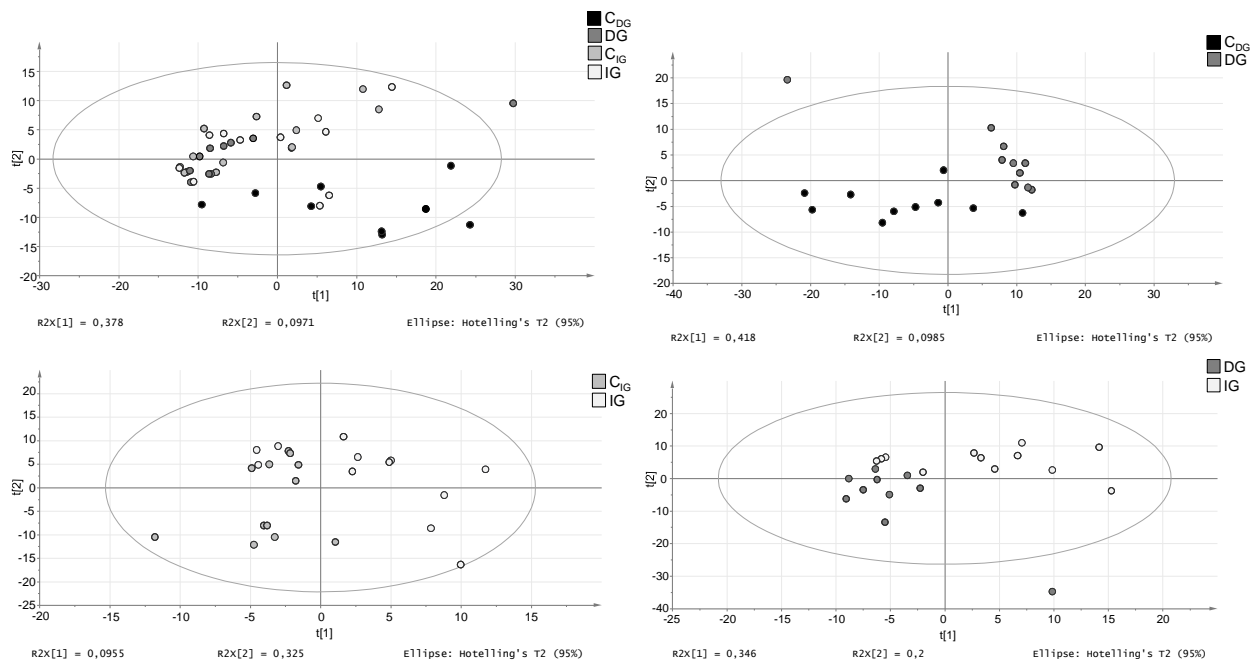


**Fig. 2.** Algal consumption of *L. spicata* by *S. scurra* after Grazing treatment in March (light grey) and in December (dark grey) after two and four days of bio-assay. DG: Direct Grazing (one frond is directly exposed to grazing by *S. scurra*); C<sub>DG</sub>: Control Direct Grazing (a frond treated as DG but without any grazer); IG: Indirect Grazing (co-incubated with the DG frond); C<sub>IG</sub>: Control Indirect Grazing (co-incubated with the C<sub>DG</sub> frond, without grazer). March and December; n = 10 per treatment. Letters shared in common between the groups indicate no significant difference (2-ways ANOVA, p<0.05, and SNK post-hoc test).

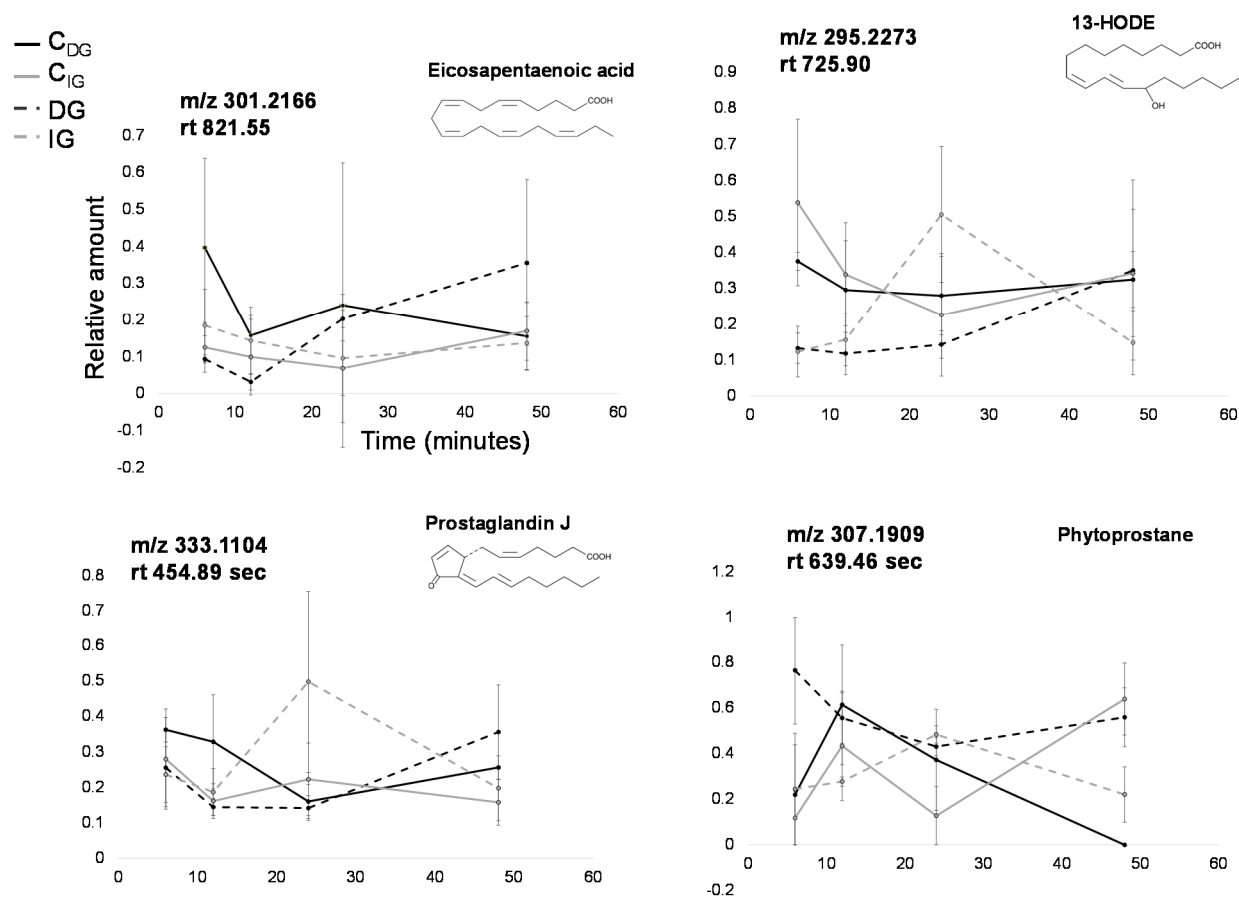


**Fig. 3.** Algal consumption of *L. spicata* by *S. scurra* after elicitation treatment in August (middle grey) and in November (dark grey), after 2 days of grazing, according to the different Elicitation treatments: E: Elicitation (one frond is directly exposed to elicitation by oligoalginates); C<sub>E</sub>: Control Elicitation (a frond treated as E but without elicitation); IE: Indirect Elicitation (co-incubated with the direct elicited alga); C<sub>IE</sub>: Control Indirect Elicitation (co-incubated with the control kelp, without elicitation). August: C<sub>E</sub>, n=9; E, n=9; C<sub>IE</sub>, n=10; IE, n=10; November: C<sub>E</sub>, n=10; E, n=10; C<sub>IE</sub>, n=10; IE, n=9.





**Fig. 4.** Results of partial least squares discriminant analysis (PLS-DA) carried out for the compounds detected in the Grazing experiment. The plot represents the variations of 514 monoisotopic peaks quantified by UPLC-MS in negative ion mode in algal samples after 6h, 12h, 24h and 48h of Grazing treatments C<sub>DG</sub> (black spots), DG (dark grey spots), C<sub>IG</sub> (light grey spots) and IG (white spots).



**Fig. 5.** Relative amounts of four annotated compounds from the endometabolome of *L. spicata* over 48 hours, according to the treatments C<sub>DG</sub> (black), DG (dark grey), C<sub>IG</sub> (middle grey) and IG (light grey). Putative corresponding compounds are indicated for each selected ion. They were annotated as eicosapentaenoic acid (EPA), 13-HODE, prostaglandin J and a phytoprostane. C<sub>DG</sub>: 6h: n=3, 12h: n=2, 24h: n=3, 48h: n=2; DG: 6h: n=2, 12h: n=3, 24h: n=2, 48h: n=3; C<sub>IG</sub>: 6h: n=3, 12h: n=3, 24h: n=3, 48h: n=3; IG: 6h: n=3, 12h: n=3, 24h: n=3, 48h: n=3. Means ± standard error.

**Table 1.** ANOVA results of season and treatment effects on algal consumption after Grazing treatment after 2 days (A) and 4 days of grazing (B).

**A**

<b>Source</b>	<b>Df</b>	<b>Sum</b>	<b>MS</b>	<b>F</b>	<b>P</b>
<b>Season</b>	1	10963	10963	0.8294	0.3655
<b>Treatment</b>	3	85599	28533	2.1586	0.1003
<b>Season*Treatment</b>	3	55244	18415	1.3931	0.2519
<b>Residuals</b>	72	951703	13218		

**B**

<b>Source</b>	<b>Df</b>	<b>Sum</b>	<b>MS</b>	<b>F</b>	<b>P</b>
<b>Season</b>	1	344831	344831	5.7549	0.01903
<b>Treatment</b>	3	388993	129664	2.164	0.09969
<b>Season*Treatment</b>	3	367141	122380	2.0424	0.11553
<b>Residuals</b>	72	4314174	59919		

## Supporting information

**S1 Fig.** Algal consumption of *L. spicata* by *S. scurra* after elicitation treatment in August and in November, after 4 days of grazing, according to the different Elicitation treatments E, C<sub>E</sub>, IE and C<sub>IE</sub>.

**S2 Fig.** Relative amounts of two ions annotated as one phytoprostane and prostaglandin B over 48 hours, according to the treatments C<sub>DG</sub>, DG, C<sub>IG</sub> and IG.

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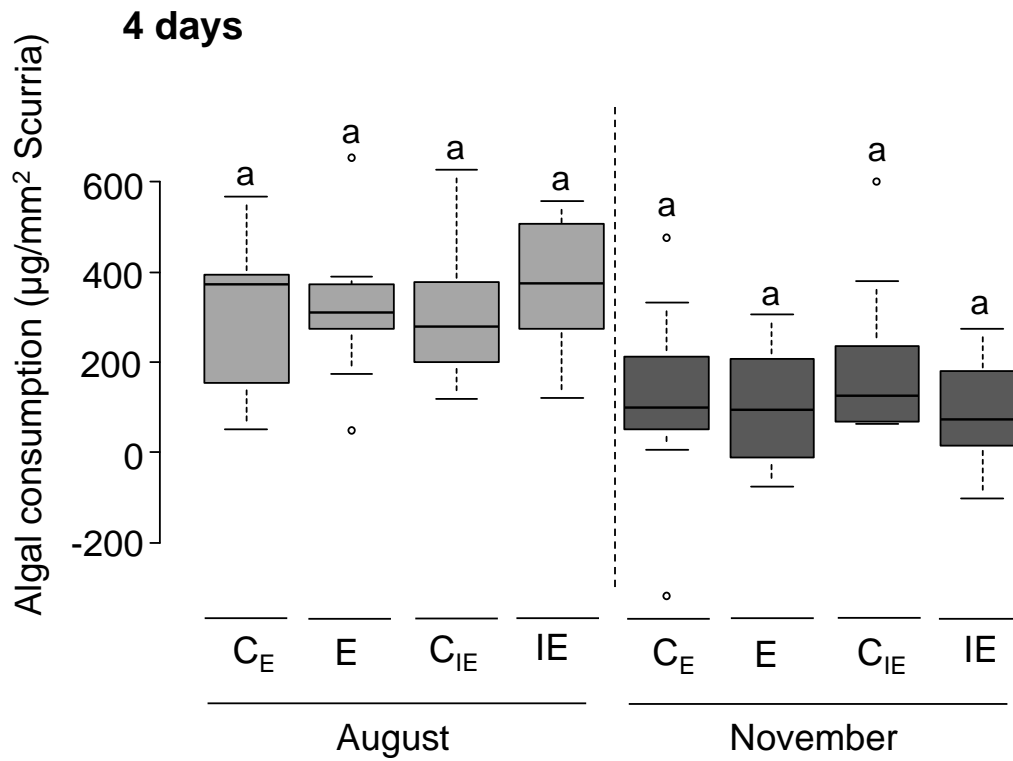
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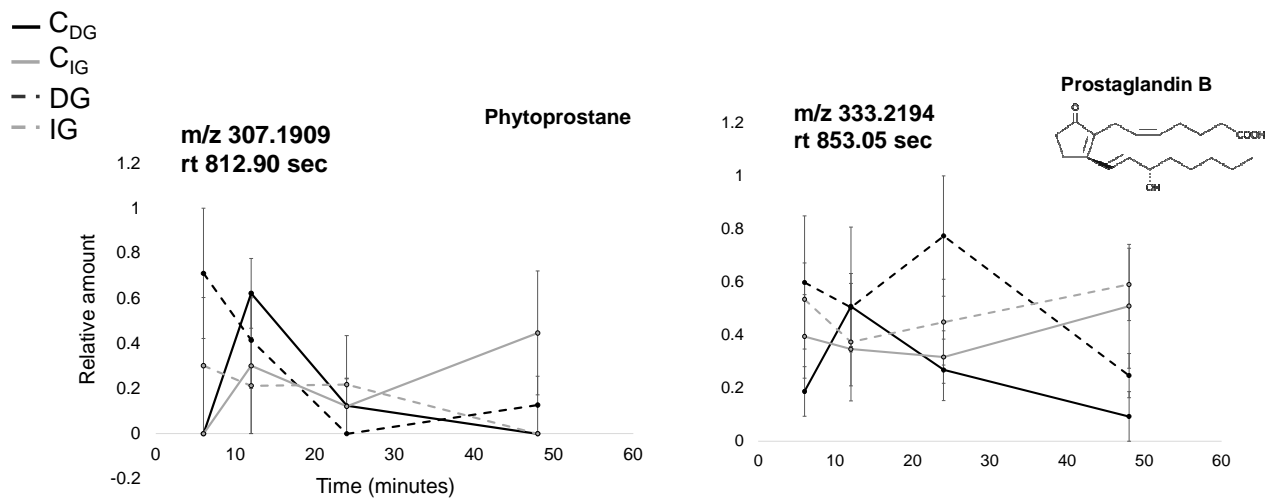
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## Supporting information



**S1 Fig.** Algal consumption of *L. spicata* by *S. scurra* after elicitation treatment in August (middle grey) and in November (dark grey), after 4 days of grazing, according to the different Elicitation treatments: E: Elicitation (one frond is directly exposed to elicitation by oligoalginates); C<sub>E</sub>: Control Elicitation (a frond treated as E but without elicitation); IE: Indirect Elicitation (co-incubated with the direct elicited alga); C<sub>IE</sub>: Control Indirect Elicitation (co-incubated with the control kelp, without elicitation). August: C<sub>E</sub>, n=9; E, n=9; C<sub>IE</sub>, n=10; IE, n=10; November: C<sub>E</sub>, n=10; E, n=10; C<sub>IE</sub>, n=10; IE, n=9.

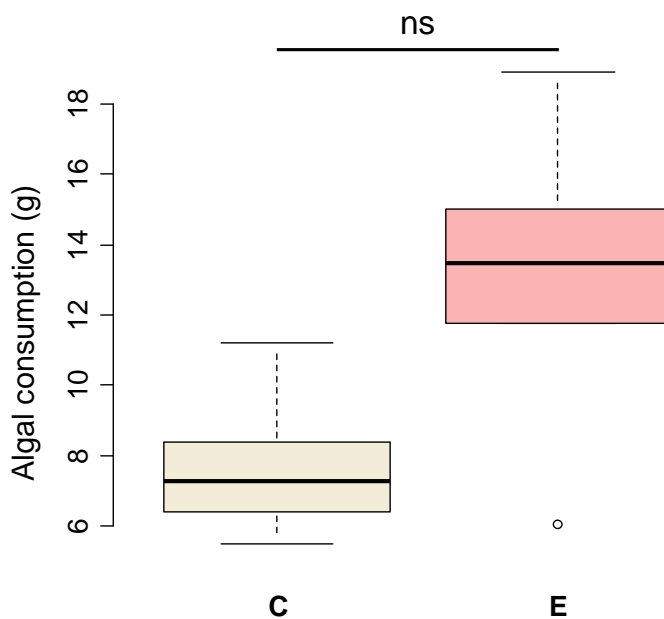


**S2 Fig.** Relative amounts of two ions annotated as one phytoprostane and prostaglandin B over 48 hours, according to the treatments C<sub>DG</sub> (black), DG (dark grey), C<sub>IG</sub> (middle grey) and IG (light grey). C<sub>DG</sub>: 6h: n=3, 12h: n=2, 24h: n=3, 48h: n=2; DG: 6h: n=2, 12h: n=3, 24h: n=2, 48h: n=3; C<sub>IG</sub>: 6h: n=3, 12h: n=3, 24h: n=3, 48h: n=3; IG: 6h: n=3, 12h: n=3, 24h: n=3, 48h: n=3. Means ± se.

## II. Impact of defense-induced kelp compounds on herbivore feeding behavior

### 1. Effects of oligoguluronate elicitation of *L. digitata* on algal consumption by a generalist herbivore

The choice test with *L. digitata* and *H. tuberculata* allowed to evaluate herbivore feeding behavior through the measurement of algal consumption by abalones. Algal mass after 2 and 4 days of grazing was subtracted to the initial algal mass in each feeder, constituting the consumption for each feeder. As two feeders of the same treatment were placed in each tank, the consumptions of the two feeders containing the same algal treatment in the same tank were added. Algal consumption according to the treatment applied to the kelps, represented in Fig 19, showed that elicited kelps tend to be more consumed by abalones than control kelps, although the difference was marginally significant (t-test,  $t = -2.27$ ,  $df = 8$ ,  $p\text{-value} = 0.053$ ).



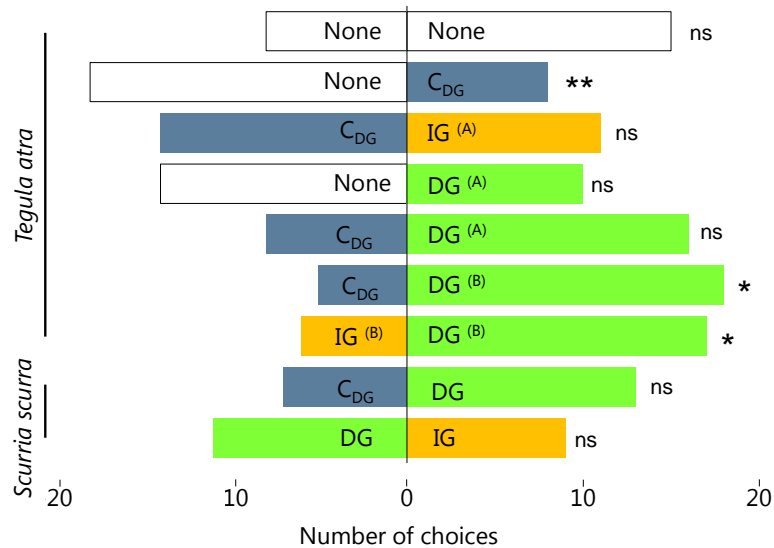
**Figure 19.** Consumption of control and elicited *L. digitata* by abalones for 4 days. ns indicates that consumption between the algal treatments is not significantly different (t-test,  $\alpha=5\%$ ).  $n=5$ .

## 2. Effects of direct and indirect grazing of *L. spicata* on attraction of a generalist herbivores

These experiments were implemented as part of Youssef Yacine's internship. Two-choice tests were performed, using two algal food sources to assess herbivore attraction for one of the two choices. Results of choices of the herbivore *T. atra* between two algal treatments are shown in Table 4 and Fig 20. Treatment (A) corresponds to 36h of grazing with a mean of 1.2 *T. atra* individuals per frond and 24h of co-incubation between DG and IG kelps, while treatment (B) corresponds to 24h of grazing with a mean of 2.5 *T. atra* individuals per frond and 24h of co-incubation between DG and IG kelps. Table 4 presents the number of choices for each option and the p-value associated with the multinomial tests performed on each experiment. The non-choice option was included in statistical tests (multinomial tests). Surprisingly, *T. atra* significantly moved more often towards the compartment with no alga (None), than the one with healthy algae ( $C_{DG}$ ). They also preferred previously grazed algae by *T. atra* with treatment <sup>(B)</sup> than the corresponding controls ( $C_{DG}$ ) and than co-incubated algae with grazed kelps ( $C_{IG}$ ) with treatment <sup>(B)</sup>. However, these differences were not found after a previous grazing by *S. scurra* and after co-incubation with kelps grazed by *S. scurra*.

It is possible to conclude from these choice experiments that there are chemical communications among the kelp and the seaweed, and that these cues modify the behavior of the generalist herbivore *T. atra*. Interestingly, the communication among kelps modifies the signal perceived by the herbivore, with a clear attraction by grazed individuals, and a possible repulsion by primed and control individuals. And finally, the communication seems herbivore-dependent, as the experiment conducted on the specialized herbivore *S. scurra* did not induce any choice for further grazing.





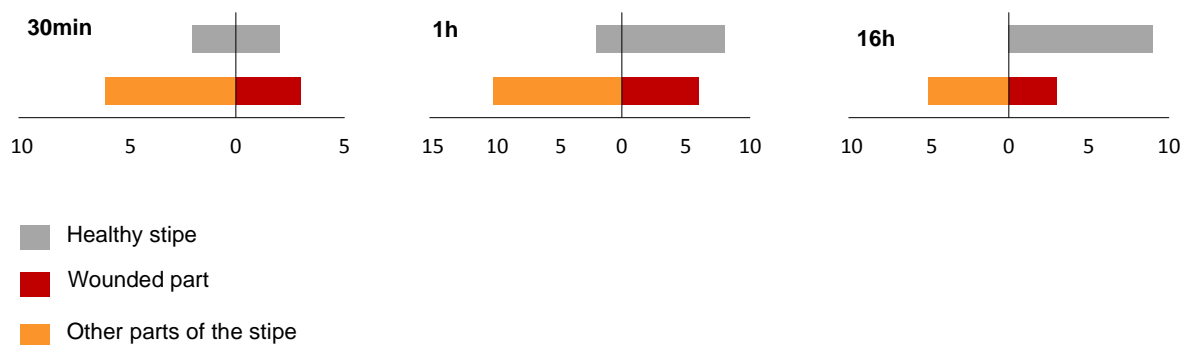
**Figure 20.** Results of the two-choice test between *L. spicata* and *T. atra* or *S. scurra* for either nothing (none) or seaweeds treated as Control Direct Grazed (C<sub>DG</sub>, a frond treated as DG but without any grazer), Direct Grazed (DG, one frond is directly exposed to grazing) or Indirect Grazed (IG, co-incubated with the DG frond). For the first choice tests, grazing and co-incubation treatments were performed using *T. atra* and for the lasts, *S. scurra* were used. Treatment (A) corresponds to 36h of grazing with a mean of 1.2 *T. atra* individuals per frond and 24h of co-incubation between DG and IG kelps, while treatment (B) corresponds to 24h of grazing with a mean of 2.5 *T. atra* individuals per frond and 24h of co-incubation between DG and IG kelps. Statistical analyses were performed using a multinomial test which considered 3 choices: choice A, choice B or non-choice. Significance of mean difference is indicated: \*\*p<0.01, \*p<0.05, NS = p>0.05, as determined from multinomial tests. n=30.

**Table 4.** Choices of *T. atra* or *S. scurra* between either nothing (none) or *L. spicata* treated as C<sub>DG</sub>, DG or IG. Treatment (A) corresponds to 36h of grazing with a mean of 1.2 *T. atra* individuals per frond and 24h of co-incubation between DG and IG kelps, while treatment (B) corresponds to 24h of grazing with a mean of 2.5 *T. atra* individuals per frond and 24h of co-incubation between DG and IG kelps. Each experiment was repeated 30 times, using a new herbivore for each choice test. Statistical analyses were performed using multinomial tests which tested the choices between choice A, choice B and non-choice.

Choice A	Choice B	Number of choice A	Number of choice B	Number of non choice	Multinomial test (p-value)
None	None	8	15	7	0.185
None	C <sub>DG</sub>	18	8	4	0.0068 **
C <sub>DG</sub>	IG (A)	14	11	5	0.1117
None	DG (A)	14	10	6	0.219
C <sub>DG</sub>	DG (A)	8	16	6	0.0724
C <sub>DG</sub>	DG (B)	5	18	7	0.0103 *
IG (B)	DG (B)	6	17	7	0.0331 *
C <sub>DG</sub>	DG	7	13	10	0.4292
DG	IG	11	9	10	0.973

### 3. Effects of natural wounds on *L. spicata* stipes on *S. scurra* attraction

As *S. scurra* inhabit the wounds that they form in natural conditions, our hypothesis was that they could be more attracted to the wound through chemical compounds coming from this damaged site. We collected wounded and healthy *L. spicata* stipes in the field, placed one *S. scurra* individual in the middle of the stipe and monitor their movement along the stipe in the lab. On wounded stipes, *S. scurra* individuals could choose between the wounding site and other locations on the stipe of *L. spicata*, while healthy stipes were used as controls to test left or right preference. Position of *S. scurra* on the stipe was noted after 30 min, 1h and 16h observation. Despite the low number of replicates ranging from 10 (30 min and 16h observation) to 20 (1h observation), herbivores did not preferentially choose the wounding site rather than other parts of the stipe (Fig. 21). Moreover, it seems that their preference for one side of a healthy stipe increased with time.



**Figure 21.** Number of observations for choices of *S. scurra* on *L. spicata* stipes. Choices on wounded stipes could be made between the wounded site and other parts of the stipe, while healthy stipes were used as controls, to test left or right preference. The herbivore was placed in the middle of the stipe and its position was noted after 30 min (n=10), 1h (n=20) and 16h (n=10).

### 4. *In situ* comparison of natural and artificial wounding effects on *L. spicata* stipes on *S. scurra* attraction

This experiment was performed as an extension of the previous one but in the field, directly involving natural conditions. Our hypothesis was that natural wounds would emit chemical compounds allowing herbivores to recognize their habitat and thus attracting them, while artificially damaged stipes would not be perceived in this way and would not be attractive for the grazer. This *in situ* experiment is a preliminary test of the attractive effect of a wound on *S. scurra* behavior, which implied a monitoring of herbivore position in wounds on *L. spicata*. Five individuals of *S. scurra* were removed from their hole during low tide, and repositioned on the kelp, either in the same hole, on an artificial wound, on the stipe close to the holdfast, or on the stipe close to the frond. Results presented in Tables 5 and 6

showed that there was no clear attraction pattern of *S. scurra* by artificial or natural wounds on *L. spicata* stipes. Observations of *S. scurra* in their wound on *L. spicata* stipes (F) showed that they all stayed in the wound during 5 days. Herbivores that were removed and put back in their wound generally stayed in their wound, suggesting no grubbing-up effect on herbivore behavior (C). After artificially wounding the stipes, a natural colonization by *S. scurra* did not seem to occur, as no herbivores were found after 1 and 5 days (B) and a grazer placed on an artificial wound did not stay in this place (A). Moreover, when a grazer was placed outside its wound on the same stipe (D) or on a neighboring stipe (E), it generally did not go back to this wound after 1 day, but one grazer could return to the hole after 5 days. We observed that removing individuals of *S. scurra* during low tide turned them unable of reattaching rapidly to kelp surface. It is likely that they require some kind of acclimation in running seawater before being manipulated. However, besides these experimental limitations, it seems that natural holes (i.e. caused by *S. scurra* themselves) are more attractive than artificial wounds. Further experiment should examine more carefully this phenomenon, as well as the determinants of the choice of a place on a stipe to dig a new hole.

**Table 5.** *In situ* treatments of *L. spicata* tested on attraction of *S. scurra*.

<b>A</b>	<b>Attraction of an artificial wounding 24h after wounding generation</b>
	5 <i>L. spicata</i> with artificial wounding made at D0 1 <i>S. scurra</i> added on the wounding at D1
<b>B</b>	<b>Control artificial wounding</b>
	5 <i>L. spicata</i> with artificial wounding made at D0 No addition of <i>S. scurra</i>
<b>C</b>	<b>Control of grubbing-up effect</b>
	5 <i>L. spicata</i> with natural wounding and 1 <i>S. scurra</i> into the wounding Removing and putting back the herbivore in the wounding
<b>D</b>	<b>Attraction of the natural wounding</b>
	5 <i>L. spicata</i> with natural wounding and 1 <i>S. scurra</i> into the wounding Removing and putting the herbivore outside the wounding, on the same stipe
<b>E</b>	<b>Natural recolonization</b>
	5 <i>L. spicata</i> with natural wounding and 1 <i>S. scurra</i> into the wounding Removing and putting the herbivore on a neighboring stipe on the same <i>L. spicata</i> individual
<b>F</b>	<b>Observation of <i>S. scurra</i> in its wounding</b>
	5 <i>L. spicata</i> without treatment

**Table 6.** Results of the in situ experiment after 1 and 5 days, for each treatment applied to the kelps.

	<b>Day 1</b>	<b>Day 5</b>
<b>A</b>	nothing on the same stipe outside the wounding nothing on the holdfast nothing	nothing basis of the stipe nothing on the holdfast nothing
<b>B</b>	nothing nothing nothing nothing nothing	nothing nothing nothing nothing nothing
<b>C</b>	into the wounding into the wounding 2 <i>S. scurra</i> into the wounding into the wounding nothing	<i>L. spicata</i> individual not found into the wounding into the wounding into the wounding nothing
<b>D</b>	basis of the stipe nothing into the wounding nothing nothing	basis of the stipe into the wounding into the wounding nothing nothing
<b>E</b>	nothing basis of the neighboring stipe nothing nothing <i>L. spicata</i> individual not found	into the wounding <i>L. spicata</i> individual not found nothing basis of the stipe nothing
<b>F</b>	into the wounding into the wounding into the wounding into the wounding into the wounding	into the wounding into the wounding into the wounding into the wounding into the wounding

## Discussion

### 1. Constraints in ecology experiments

All the experiments of this chapter share the common constraint of biological variability and influence of uncontrolled environmental conditions usually faced in ecology experiments. For preliminary and exploratory experiments (experiments 3. and 4.), the low number of replicates generally did not allow to draw solid conclusions, as statistical tests could not be applied. The *in situ* experiment on attraction of *S. scurra* by *L. spicata*'s wounds was performed on only 5 individuals of *L. spicata* per treatment. Moreover, as this experiment was performed in the field, many parameters could not be controlled, such as the effects of the tide and the waves or the ambient temperature, and the likely requirements of high tide conditions. This experiment should thus be repeated several times in order to get a better estimation of the effects of algal wounds on herbivore attraction. In non-choice feeding assays, the algal biological variability was added to the season effect, but mostly by the biological variability due to the herbivore (genetic variability, age, appetite, biological rhythm possibly influenced by the rhythm of tides,...) and especially to the interaction between the herbivore and its algal host. Thus, biological replicates have to be numerous in order to explicitly take into account the interaction of these parameters on the effects of direct and indirect grazing, and algal-derived distance signaling.

If results showed that *T. atra* was clearly attracted to already grazed *L. spicata* by conspecifics for 2 days, but lab and field experiments testing *S. scurra* attraction for natural or artificial wounds on kelp stipes did not give evident conclusions. These experiments did not show a clear attraction of *S. scurra* for artificial and natural wounds. *S. scurra* did not seem to recognize a natural wound, but seemed to stay in the wound that it shaped itself. The chemical compounds of the grazed tissues inside the wound could thus be very localized and internalized without being diffused in the surrounding environment. This hypothesis could explain the absence of natural recolonization of artificial wounds during the experiment, as well as the absence of new holes in stipes already colonized by a *S. scurra*.

### 2. Effects of direct and indirect grazing on consumption by grazers and on algal metabolic regulations

Despite this variability, some conclusions can be noted on deterrence of *L. spicata* on *S. scurra* and the metabolic regulations into algal tissues. Results on consumption of *L. spicata* by herbivores in non-choice bio-assays showed that both the algal physiology and the activity of grazers were different according to the season. Indeed, algal weight gain was higher in November while consumption by herbivores increased in March. This seasonal effect could be explained by the upwelling occurring between September and January, which provide high amounts of nutrients and fresh water are provided to the surface, possibly leading to a higher growth rate and resistance to herbivores compared with the

non-upwelling season (from February to August). Moreover, algal resistance can also be modified, as the chemical composition of algal tissues was already shown to change according to the period of the year.

Analyses of the endo-metabolome of *L. spicata* allowed to discriminate a grazed alga from its co-incubated neighbor, and from an ungrazed healthy alga over 48h. Thus, metabolic modifications occur not only in algal tissues upon grazing but also, and differently, after co-incubation with a grazed alga. Further studies allowed to annotate some compounds as fatty acids and oxylipins, suggesting that the fatty acid metabolism was activated and regulated upon grazing and co-incubation with a grazed kelp, and that the co-incubated kelp could possibly perceive a chemical signal from the grazed kelp. However, these annotation hypotheses still have to be verified through LC-MS/MS analyses and comparison with chemical standards.

### 3. Effects of defense-induced kelp compounds on herbivore feeding behavior

Complementary experiments on herbivore preference upon grazing stress in kelps could highlight that previously grazed or elicited algae were preferred for generalist herbivores compared to ungrazed or control algae, for both *L. digitata* and *L. spicata*. This result first showed that herbivores were able to perceive different signals coming from stressed algae and control algae, then choosing the stressed kelps. These chemical compounds emitted following a stress (elicitation or grazing) could therefore allow herbivores to better locate an edible food source, i.e. previously consumed. Our results were also consistent with previous observations on *A. nodosum* and *L. japonica* which responded differently according to the herbivore, changing their metabolic and ecological responses (Pavia and Toth, 2000; Molis et al., 2008). Our results could appear inconsistent with previous ones performed on the Fucale *F. vesiculosus*. In this case, two-choice feeding assays showed that ungrazed *F. vesiculosus* were significantly preferred to grazed algae by the generalist isopod *Idotea baltica* and by *Littorina obtusata*, but only after 9 and 15 days, respectively, of previous grazing (Flöthe et al., 2014). Feeding bio-assays showed that previously grazed *F. vesiculosus* were less palatable compared with non-grazed controls. However, the timing of our experimental set-up was significantly shorter, with a previous grazing period lasting only 2 days. Interestingly, in similar conditions (3 days of previous grazing), both *I. baltica* and *L. obtusata* showed a slight preference towards fresh grazed *Fucus* (Flöthe et al., 2014). Thus, responses to grazing are not universal as they strongly depend on the herbivore and algal species, and also vary according to time and season. There is still a lack of studies in order to understand the effect of each parameter which can favor or deter the herbivore.

Our two-choice bio-assay also suggested that the generalist herbivores *T. atra* preferred previously grazed *L. spicata* compared to their co-incubated neighbors. This observation could be

explained by the stronger recognition of freshly grazed algae by herbivores, letting the intact neighboring algae (indirect grazed) imperceptible for them. Another hypothesis would be the deterrent perception of the indirect grazed kelps by the herbivore, leading herbivores to choose grazed kelps. However, according to our results on metabolic regulation upon grazing pressure (Chapter I and II), a short grazing period (up to 2 days) was sufficient to induce *de novo* biosynthesis of compounds, such as fatty acids and oxylipins, that could be attractive for neighboring herbivores. On the contrary, a longer grazing stress (more than one week) would lead to the production of deterrent compounds and anti-herbivory protection, as shown in *Fucus* (Flöthe et al., 2014). This early attractive effect could also depend on the type of herbivore: *T. atra* seemed to recognize kelps that were grazed by its own species, while not discriminating kelps grazed by the specialist herbivore *S. scurra*. This specific recognition could be linked to a chemical recognition of grazed *L. spicata* according to the herbivore species or the relationship between each herbivore species and this algal species. Thus, depending on the fact that the herbivore is either generalist or specialist of *L. spicata*, on the algal species (for instance between Laminariales and Fucales) and on the timing of algal induction, the chemical signature of compounds emitted by a direct grazed or an indirect grazed kelp could be different.







## **Chapter III**

Aldehyde-based signaling and defense of kelps  
against herbivores



## Introduction

In response to grazing by herbivores, Eukaryotes evolved defense mechanisms of innate immunity. These comprise the recognition of elicitors through innate receptors, followed by a transient oxidative burst, involving the activation of the membrane-associated enzyme NADPH oxidase and a rapid apoplastic production and accumulation of reactive oxygen species (ROS) like  $O_2^-$  and  $H_2O_2$  in the surrounding environment (Conrath et al., 2002). These first steps of innate immunity, also present in algae, allow recognition between self (or Microbe-Induced Molecular Patterns; MIMPs) and non-self (or Microbe-Associated Molecular Patterns; MAMPs) and lead to defense responses (Weinberger, 2007). In the kelp *L. digitata*, specific derived products from alginates, the oligoguluronates (GG), constitute MIMPs and were shown to trigger an oxidative burst including a release of  $H_2O_2$ , which had anti-microbial effects (Küpper et al., 2001). Oligoguluronates were also shown to induce a protection of the kelp from infection by the endophyte *Laminariocolax tomentosoides* within 7 days, when kelps were previously elicited (Küpper et al., 2002).

Moreover, mechanisms of innate immunity in Eukaryotes include the release of free fatty acids and their oxidized derivatives, named oxylipins. Brown macroalgae produce oxylipins from C18 and C20 fatty acids, which could be involved in algal defense (Potin et al., 2002; Pohnert, 2004). Treatment of *L. digitata* thalli with lipopolysaccharides (LPS; cell wall extracts from bacteria) from *Salmonella abortus equi* was shown to induce the production of free fatty acids and oxylipins (Küpper et al., 2006). Moreover, jasmonic acid was described in vascular plants after derivatization of C18 and C20 oxylipins. In an evolutionary point of view, it is likely that jasmonate signaling have evolved between the primary and the secondary endosymbiotic events and is therefore considered as a putative common ancestral trait of photosynthetic Eukaryotes (Bouarab et al., 2004). Most of studies on biosynthetic pathways of the fatty acid and their oxidized derivatives and their involvement in algal defense against herbivores were conducted in microalgae belonging to the Stramenopiles lineage, the diatoms. Several aldehydes, produced enzymatically by lipoxygenases (LOX) and hydroperoxide lyases (HPL), were found to decrease the reproduction success and egg viability of copepods that grazed on diatoms (Pohnert, 2004). In macroalgae, fatty acid derivatives originating from the oxylipin pathway were shown to have attracting roles as sexual pheromones in brown algae (Pohnert and Boland, 2002), as well as defensive roles in red algae against epiphytes (Lion et al., 2006), endophytes (Bouarab et al., 2004) and herbivores (Rempt et al., 2012).

In the kelp *Laminaria angustata*, aldehydes are also produced enzymatically. C6 aldehydes are derived either from C18 or from C20 fatty acid and C9 aldehydes are exclusively formed from the C20 fatty acid, arachidonic acid (Boonprab et al., 2003a; Boonprab et al., 2003b). In *L. digitata*, Küpper et al. (2006) found that free fatty acids and oxylipins were produced upon application of LPS, and it was then demonstrated that a wide range of aldehydes were emitted by this kelp upon elicitation by

oligoguluronates (Goulitquer et al., 2009; Thomas et al., 2011) and that copper stress also induced a strong release of fatty acids, oxylipins and amino acids (Goulitquer et al., 2009; Ritter et al., 2014). Moreover, chemical analyses conducted *in situ* above kelp beds revealed emissions of aldehydes reaching a peak during and after low tide (Goulitquer et al., 2009).

In this chapter, we assessed the roles of aldehyde-based signaling in the context of kelp/herbivore interactions. We investigated the effects of aldehyde signals on both algal consumption by herbivores and on algal metabolism. The first part of this chapter is devoted to the article, which deals with the effects of aldehyde signaling on the interaction between the kelp *L. digitata* and its specialist herbivore *P. pellucida*. We evaluated their effects on algal consumption by their grazers, then on algal physiology by measurement of the oxidative burst and finally the metabolic regulations that they induce in algal tissues through metabolomic approaches. The second part of the chapter consists in complementary studies which evaluated the effects of aldehyde-based signaling on the interaction between the kelp *L. spicata* and the generalist herbivore *T. atra*.

# I. Exploring the role of aldehyde-based signaling during kelps/herbivores interactions

## General outline

In order to investigate the effects of aldehyde signaling during kelp/herbivore interactions, we applied combined approaches of metabolomics and bio-assays with the kelp *L. digitata* and its specialist herbivore *P. pellucida*. We first evaluated the effects of incubation of laboratory-grown algae in aldehydes on algal consumption by their grazers. Then, we assessed their impacts on algal physiology by measurement of the oxidative burst. We finally analyzed the metabolic regulations that they induced in algal tissues over 24h after incubation. Results showed that aldehyde incubation induced different effects on later algal consumption by herbivores according to chemical nature, i.e. 4-HHE (C6-aldehyde), 4-HNE (C9-aldehyde) or dodecadienal (C12-aldehyde), and concentration of aldehyde, from  $1 \mu\text{g.mL}^{-1}$  to  $10 \text{ ng.mL}^{-1}$ . This suggests that aldehyde perception could rapidly affect algal metabolism through different regulation pathways. Moreover, these aldehydes induced metabolic modifications in algal tissues compared to control algae, especially a production of FFAs and their oxidized derivatives, oxylipins. These modifications occurred differently according to time over one day, between early and late time periods. Thus, the results suggest that the perception of specific aldehydes could have a major ecological role on the interactions between kelps and their herbivores.



## Article

# Exploring the role of aldehyde-based signaling during kelps/herbivores interactions

**Authors:** Cabioch L.<sup>1,2,3</sup>, Desrut A.<sup>1,2</sup>, Dartevelle L.<sup>1,2</sup>, Potin P.<sup>1,2</sup>, Faugeron S.<sup>3,4</sup>, Leblanc C.<sup>1,2</sup>

### **Affiliations:**

<sup>1</sup> Sorbonne Universités, UPMC Univ Paris 06, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France

<sup>2</sup> CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique, Roscoff, France

<sup>3</sup> Centro de Conservación Marina and CeBiB, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

<sup>4</sup> Sorbonne Universités, UPMC University Paris 06, CNRS, UMI 3614, Evolutionary Biology and Ecology of Algae, Station Biologique, Roscoff, France

Article in preparation



# Exploring the role of aldehyde-based signaling during kelps/herbivores interactions

Cabioch L.<sup>1,2,3</sup>, Desrut A.<sup>1,2</sup>, Darteville L.<sup>1,2</sup>, Potin P.<sup>1,2</sup>, Faugeron S.<sup>3,4</sup>, Leblanc C.<sup>1,2</sup>

<sup>1</sup> Sorbonne Universités, UPMC Univ Paris 06, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France

<sup>2</sup> CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique, Roscoff, France

<sup>3</sup> Centro de Conservación Marina and CeBiB, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

<sup>4</sup> Sorbonne Universités, UPMC University Paris 06, CNRS, UMI 3614, Evolutionary Biology and Ecology of Algae, Station Biologique, Roscoff, France

## Abstract

In the terrestrial environment, volatile organic compounds, such as aldehydes, are emitted during defense responses by plants against herbivores and perceived by neighboring plants as warning distance signals, leading to priming. In the marine environment, similar distance signaling, based on water-borne cues, also exists during interactions between macroalgae and herbivores, but their biological and ecological roles remain unclear. In brown algae, chemical elicitation or grazing induces regulations of transcription and metabolic pathways, as well as emission of chemical compounds like aldehydes. In laboratory, upon stress conditions, the brown alga *Laminaria digitata* emits a wide range of volatile aldehydes, but their biological roles as potential defense signals remain unknown in this alga in response to grazing. In this context, bioassays using the limpet *Patella pellucida* and *L. digitata* from North-Atlantic Brittany are used for determining the effects of algal incubation with 4-hydroxyhexenal (4-HHE), 4-hydroxynonenal (4-HNE) and dodecadienal on algal consumption by grazers. Simultaneously, we have developed metabolomics approaches to study algal metabolic modifications after treatments of *L. digitata* with these aldehydes. The results displayed that, unlike treatment of the kelps with 4-HNE or dodecadienal, treatment with 4-HHE decreases algal consumption by herbivores, only at 100 ng.mL<sup>-1</sup>. Moreover, we showed that algal metabolome is modified according to the type of aldehyde, especially concerning fatty acid degradation pathways. As kelp beds constitute complex ecosystems with roles of habitat and food source for marine herbivores, the perception of specific aldehydes could have a major ecological role on the structuration of marine kelps/grazers communities.

## Introduction

In response to pathogen or herbivore attacks, land plants developed capabilities to set up defenses, locally at the point of the infection or of grazing pressure but also at distance by the emission and perception of volatile chemical signals either by the other parts of the plant or by neighboring plants. Other photosynthetic eukaryotic lineages, including red and brown algae, share ancestral innate immunity mechanisms with the Plant lineage, such as the recognition of biotic challenges by detecting Microbe-Induced Molecular Patterns (MIMPs) or Microbe-Associated Molecular Patterns (MAMPs) (Weinberger, 2007). This detection triggers an oxidative burst, corresponding to the activation of the plasma membrane-associated NADPH oxidase and the accumulation of reactive oxygen species (ROS) in the surrounding environment (Conrath et al., 2002), that has also been detected in animals (Torres and Dangl, 2005) and brown algae, such as *Laminaria digitata* in response to oligogulonates elicitation (Küpper et al., 2001). In this species, the oxidative burst has shown anti-microbial roles, and is involved in the establishment of infection resistance against endophytes (Küpper et al., 2002).

In Eukaryotes, another conserved trait in innate immunity processes is the accumulation and release of fatty acids and oxylipins during defense responses. In land plants, the generation of oxidized fatty acid derivatives proceeds from oxygenation of free polyunsaturated fatty acids (PUFAs) mostly by intervention of lipoxygenases (LOXs), which generate hydroperoxides, until production of aldehydes by hydroperoxide lyases (HPLs) (Feussner and Wasternack, 2002). The derivatives of C18 (octadecanoids) and C16 (hexadecanoids) fatty acids such as salicylic acid, jasmonic acid and ethylene are known as defense hormones involved in intra- or interplant distance signaling (Fu and Dong, 2013; Meng and Zhang, 2013). Marine macroalgae were found to generate C20 and C18 PUFAs and oxidized derivatives during innate immunity responses (Potin et al., 2002; Bouarab et al., 2004). In response to bacterial MIMPs, the kelp *L. digitata* induced a rapid release of FFAs with a simultaneous accumulation of oxidized derivatives of linolenic (C18:2) and eicosapentaenoic acids (C20:5), and oxylipins like 13-hydroxyoctadecatrienoic acid (13-HOTrE) and 15-hydroxyeicosapentaenoic acid (15-HEPE) (Küpper et al., 2006). In this species, the fatty acids linolenic acid (C18:3) and arachidonic acid (C20:4) and methyl jasmonate (MeJA) were also shown to induce an oxidative burst and a production of a number of FFAs, leading to a protection against algal endophytes (Küpper et al., 2009). In *L. angustata*, aldehydes and hydroperoxides were constitutively present in algal tissues, such as C9- and C6-aldehydes (Boonprab et al., 2003a; Boonprab et al., 2003b; Boonprab et al., 2006), whereas diverse C6-, C7-, C8- and C9-aldehydes, such as 4-HHE and dodecadienal, were strongly released into the surrounding seawater following elicitation by oligogulonates or in response to oxidative copper stress (Goullitquer et al., 2009; Thomas et al., 2011). During low tides, aldehyde amounts in seawater and air drastically increased above kelp beds, highlighting specific emissions of C6- and C9-aldehydes (Goullitquer et al., 2009). Presence of water-borne cues present in seawater surrounding kelps at low tides was shown later to modify defense responses upon elicitation in young *L. digitata* (Thomas et al., 2011). In diatoms,

poly-unsaturated aldehydes (PUAs), biosynthesized from cell membrane-derived free fatty acids, have been shown to have a strong biological effect on their herbivores, copepods (Miralto et al., 1999; Pohnert and Boland, 2002; Ianora et al., 2003).

Kelp beds are natural sources of a wide range of aldehydes, for which the ecological and biological roles have been hardly explored. In this context, we investigated their potential roles as distance defense signals during interactions between kelps and their herbivores. Thus, we explored the effects on algal protection against herbivores of three specific aldehydes, namely 4-hydroxy-hexenal (4-HHE), 4-hydroxy-nonenal (4-HNE) and dodecadienal that were previously shown to be strongly induced after diverse abiotic and biotic stresses in *L. digitata* (Goulitquer et al., 2009). To reach this goal, we monitored the effects of incubation of *L. digitata* with aldehydes on subsequent algal consumption by its specialist herbivore *Patella pellucida*. We also studied the impacts on algal physiology and metabolism, by following oxidative responses and by analyzing the modifications of the algal endometabolome via metabolic profiling approaches.

## **Materials and methods**

### **Algal material and herbivore sampling**

All experiments were performed using young macroscopic diploid sporophytes of *Laminaria digitata*. Unialgal cultures of *L. digitata* sporophytes were obtained from random crosses of gametophyte batches. The latter were raised in the laboratory from mature wild sporophytes collected in the same population. Two weeks after crossing, the sporophytes were transferred to 10 L flasks and grown until they reached a size of about 3 to 4 cm, in Provasoli Enriched Seawater (PES) culture media prepared with natural filtered seawater (FSW), collected off shore of Roscoff, as described in Thomas et al. (2011). Algal cultures were illuminated with daylight-type fluorescent lamps at an irradiance of  $25 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 10h per day and kept at  $13\pm 1^\circ\text{C}$ .

The blue-rayed limpets *Patella pellucida* of 3-4 mm length were collected either at low tide or by diving, on *L. digitata* fronds. In laboratory, grazers were kept in an aquarium, and fed on *L. digitata* fronds until bioassay. Running seawater was constantly supplied and chilled at  $15\pm 1^\circ\text{C}$ , with constant air bubbling in half-light conditions.

### **Aldehyde and elicitation treatments**

Stock solutions of ( $\pm$ )-4-hydroxy-2E-hexenal (4-HHE;  $10 \text{ mg}\cdot\text{mL}^{-1}$ ) and ( $\pm$ )-4-hydroxy-2E-nonenal (4-HNE;  $10 \text{ mg}\cdot\text{mL}^{-1}$ ) from Interchim were prepared in EtOH 100% and the solution of 2,4-Dodecadienal ( $20 \text{ mg}\cdot\text{mL}^{-1}$ ) from Sigma was dissolved in distilled water. For one aldehyde exposure, *L. digitata* plantlets were incubated in 50 mL (4 individuals) or in 70 mL (8 individuals) of FSW for 4h at

13°C under continuous agitation. 4-HHE (H treatment), 4-HNE (N treatment), Dodecadienal (D treatment) were added to the plantlets after dilution in 10 µL of EtOH 70%, to obtain a final concentration of 10 ng.mL<sup>-1</sup> to 1 µg.mL<sup>-1</sup> according to the aldehyde and the experiment. The control algae were treated in the same way, by adding only EtOH 70% in filtered seawater.

Alginate oligogulonates (poly-alpha-1,4-L-guluronic acid blocks, GG blocks) were prepared by acid hydrolysis according to Haug et al. (1974), using sodium alginate from *Laminaria digitata* stipes (Danisco, Landerneau, France). Elicitation treatment was performed by placing one *L. digitata* plantlet individually in 30 mL FSW with GG at a final concentration of 150 µg.mL<sup>-1</sup> for 1 hour (E treatment) at 13°C under agitation. Its control was treated equally, with seawater instead of GG solution.

For metabolomic analyses, each aldehyde treatment (H, N, D) was performed at 100 ng.mL<sup>-1</sup> with 12 plantlets incubated together during 4h, rinsed and put individually in 30 mL FSW under agitation. Four plantlets (corresponding to 4 pseudo-replicates) were frozen in liquid nitrogen after 4h incubation in aldehydes (4h time), and 4h (8h time) and 24h (28h time) after incubation in FSW. For elicitation treatment (E), the individual elicited and control plantlets were rinsed after 1h, put in 30 mL FSW under agitation and freeze-dried after 3h (4h time) or after 24h (28h time).

Before bioassays with herbivores, algal treatments were performed using 8 plantlets incubated together with an aldehyde at different concentrations for 4h, or with GG solution at 150 µg.mL<sup>-1</sup> for 1h. Treated and control algae were then rinsed twice in FSW and placed individually in 30 mL fresh FSW for 24h under agitation.

### **Hydrogen peroxide measurements**

The concentration of hydrogen peroxide in the medium around algae was determined using the luminol chemiluminescence method (Glazener et al., 1991) with a Tristar luminometer (EG&G Berthold, Bad Wild-bach, Germany). During Elicitation (E) and Aldehyde treatments (H, N, D) performed at 100 ng.mL<sup>-1</sup>, 150 µL of seawater were taken for one measurement, just before incubation in aldehyde or in GG, and then every 5 min during 1h after addition of aldehyde or GG solutions. Aliquots were put in 96-well microplates (Lumitrac 200, White, Greiner Bio-One). In the luminometer, 50 µl of 20 U.mL<sup>-1</sup> horse- radish peroxidase (Boehringer Mannheim, Meylan, France), dissolved in pH 7.8 phosphate buffer, and 100 µl of 0.3 M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) solution were added to the sample. Chemiluminescence was recorded immediately after the last injection with a signal integration time of 0.55 s. The concentration of H<sub>2</sub>O<sub>2</sub> in nmol.g.FW<sup>-1</sup> was calculated for each sample based on a standard calibration curve. The H<sub>2</sub>O<sub>2</sub> production was estimated by integrating the total amount of H<sub>2</sub>O<sub>2</sub> monitored over one hour. The experiment was repeated 4 times for each treatment and 1-way ANOVA was performed, followed by Student-Newman-Keuls (SNK) post-hoc tests for comparisons of the mean production, using the free software R.

### **Bioassays of algal consumption by grazers**

For this ‘no-choice’ feeding based bioassay, 5 *L. digitata* plantlets were submitted to grazing by *P. pellucida*, while the 3 others were used to control autogenic changes of mass during experiment. Six *P. pellucida* were placed on one plantlet in 50 mL filtered seawater at 13°C for 6 days. Algal mass was measured at the beginning of bioassay before consumption, 3 days and 6 days after exposition to grazing or to nothing for control algae. The experiment was repeated 25 times for GG treated algae, and 5, 10 or 15 times according to the type of aldehyde and to the concentration. The data were analyzed using Kruskal-Wallis tests for comparison of mean consumption between the different treatments, for each aldehyde treatment, using the free software R.

### **Metabolomic analyses and metabolic profiling of FFAs and their oxygenated derivatives**

For each algal treatment (H, N, D, E) and sampling time (4, 8 and 28h), metabolic extracts were obtained from 100 mg of frozen algal powder with 1 mL MeOH:H<sub>2</sub>O (8:2), as described in Ritter et al. (in preparation). In each sample, 1.25 µg.mL<sup>-1</sup> 12-OH-lauric acid was added as an internal standard for analysis in the negative mode. To separate FFAs and their derivatives from polar compounds, samples were first dried with liquid nitrogen at 40°C and metabolic extraction was made with 200 µL of Ethyl acetate and 200 µL H<sub>2</sub>O. The ethyl acetate phase was recovered and dried with liquid nitrogen at 40°C. For metabolomic analyses, 90 µL EtOH were added to each sample and aliquots of 50 µl of each extract were used for analyses. Chemical compounds were first separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an Electro Spray Ionization (ESI) source running on the negative ionization mode, as described in Ritter et al. (2014). Metabolic samples were analyzed using a 1.9 µm Thermo Hypersil Gold C18 column (100 x 2.1 mm) and the mobile phase A was composed of 0.1% acetic acid in H<sub>2</sub>O, and mobile phase B was 0.1% acetic acid in acetonitrile. The gradient, starting at 0% B, consisted of an initial hold at 60% mobile phase B from 1 to 3 min, followed by a gradient to 95% B in 12 min and a hold for 4 min, followed by re-equilibration for 4 min at 0% B, for a total run time of 23 min. Xcalibur 2.1 software was used for instrument control and data acquisition.

Mass spectra data were processed by XCMS on the online version (Galaxy-Workflow4metabolomics ; Giacomoni et al., 2014), after conversion of raw spectra to mzXML format. Data processing was performed using centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3,100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples necessary at 0.7. For correction of retention time, the obiwarp method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 20, and 10 for the third group step. Fillpeaks step was used with the chrom filling method. Finally,

annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of  $m/z$  values. The final table contained ion relative abundances in each sample, which corresponded to peak areas normalized by the standard peak area in the same sample. Multivariable statistical analyses were performed using Partial Least Squares - Discriminant Analysis (PLS-DA), and Number of MisClassifications (NMC) and p-value of PLS-DA were obtained, using R. Then, extraction of the 10 most discriminant ions using Variable Importance in Projection (VIP) and putative annotation of some of those ions were made based on previous studies (Ritter et al., 2008).

For the chemical identification of selected compounds, complementary analyses were carried out on the mass spectrometry facility of Corsaire-Laberca (Nantes), using a high performance liquid chromatography (HPLC) system (Ultimate® 3000 Dionex, Thermo Fisher Scientific). In this purpose, the following chemical standards from Interchim were used: jasmonic acid, 9(S)-hydroperoxyoctadecatrienoic acid (9(S)-HpOTrE), 13(S)-hydroperoxyoctadecatrienoic acid (13(S)-HpOTrE), 20-hydroxy-leukotriene B4 and stearidonic acid. The chromatographic separation was performed on Hypersil Gold C18 column (2.1 mm × 100 mm, 1.9 μm particle size, Thermo Fisher Scientific). Mobile phase consisted in water (A) and acetonitrile (B) both containing 0.1 % acetic acid (A). The used elution gradient (A:B, v/v) was as follows: 100:0 from 0 to 1.0 min; 40:60 at 3 min; 5:95 at 15 min for 4 min then 100:0 for 4 min. The injected volume was 5 μL, the flow rate was 350 μL.min<sup>-1</sup> and the temperature of the column was maintained at 35 °C. The LC system was coupled to a Hybrid Quadrupole-Orbitrap Mass Spectrometer (Q Exactive™, Thermo Fisher Scientific) with a heated electrospray ionization source (HESI). Nitrogen was used as sheath gas and auxiliary gas at flow-rates of 55 and 10 a.u (arbitrary units), respectively. The ion transfer tube temperature was set at 350°C, the vaporizer temperature at 300°C and the electrospray voltage was set at 3.0 kV in positive mode and -3.0 kV in negative with a s-lens RF level of 50%. For fullMS acquisition a polarity switching ion mode (positive/negative) with a mass range of  $m/z$  65-975 at a mass resolving power of 70 000 Full Width Half Maximum (FWHM at 200  $m/z$ ) are used. The automatic gain control target and maximum injection time were 1e6 counts and 100 ms, respectively. The normalized collision energy (NCE) used for targeted MS/MS and data dependent MS/MS modes was 35. Each target  $m/z$  was monitored with a 40 seconds window (retention time ± 20 seconds), 2-amu isolation window (target  $m/z$  ± 1 amu). For injection, the extract was diluted by 2 in ACN:H<sub>2</sub>O (50:50) and 5 μL were injected the same day on the LC-HRMS system.

## Results

### Aldehyde incubation showed different effects on later algal consumption by herbivores according to chemical nature and concentration

The effects of aldehydes on algal defense responses against herbivores were first assessed by measuring consumption of *L. digitata* tissues by *P. pellucida* during 3 days (Fig 1), following a 4-hour-incubation in different concentrations of aldehydes and 24 hours in FSW. Oligoguluronate (GG) elicitation of algae was tested in similar conditions. Consumption of elicited algae was higher than consumption of control algae, but not significantly ( $W = 248$ ,  $p$ -value = 0.22). Dodecadienal increased significantly consumption by herbivores at  $100 \text{ ng.mL}^{-1}$  compared to control ( $W = 24$ ,  $p$ -value = 0.02). 4-HNE did not induce significant changes in consumption by herbivores at  $1 \text{ }\mu\text{g.mL}^{-1}$  ( $W = 14$ ,  $p$ -value = 0.84) and  $100 \text{ ng.mL}^{-1}$  ( $W = 17$ ,  $p$ -value = 0.42). However, effects of 4-HHE on algal consumption depended on the concentration of this aldehyde applied to the alga. Over  $500 \text{ ng.mL}^{-1}$ , 4-HHE had no significant effect on algal consumption by *P. pellucida* ( $W = 22$ ,  $p$ -value = 0.06 and  $W = 17$ ,  $p$ -value = 0.42 for  $1 \text{ }\mu\text{g.mL}^{-1}$  and  $500 \text{ ng.mL}^{-1}$  respectively). Interestingly, at the concentration of  $100 \text{ ng.mL}^{-1}$ , 4-HHE decreased significantly grazing by herbivores (Wilcoxon test:  $W = 52$ ,  $p$ -value = 0.01). At lower concentrations, 4-HHE had no effect, neither at  $50 \text{ ng.mL}^{-1}$  ( $W = 13$ ,  $p$ -value = 1), nor at  $10 \text{ ng.mL}^{-1}$  ( $W = 46$ ,  $p$ -value = 0.79).

### Aldehydes do not trigger an oxidative burst in *L. digitata* plantlets

As already established for *L. digitata* (Küpper et al., 2001), the amount of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) was significantly increased over 60 minutes, in seawater surrounding *L. digitata* culture plantlets, after elicitation by oligoguluronates (GG), compared to control, showing the occurrence of a rapid oxidative burst. On the contrary, no significant difference of  $\text{H}_2\text{O}_2$  accumulation was recorded in surrounding seawater of algae after addition of one of the three aldehydes, compared to control (Fig 2; ANOVA F-value = 19.98,  $p$  value < 0.001, SNK test: C=H=N=D<E). Elicitation by GG triggered  $\text{H}_2\text{O}_2$  production of 3 to 4 times more than that measured in control *L. digitata* or treated with 4-HHE, 4-HNE or dodecadienal.

### Aldehyde treatments induced metabolic modifications in algal tissues compared to controls

Partial least squares discriminant analyses (PLS-DA) were carried out on the 177 ions detected by LC-MS analyses (Fig 3). The statistical post-tests were not conclusive at each sampling time (PLS-DA: 4h: NMC = 0.69,  $p$ -value = 0.18; 8h: NMC = 0.82,  $p$ -value = 0.76; 28h: NMC = 0.78,  $p$ -value = 0.51). This could reflect a high biological variability of the metabolic responses, avoiding to statistically discriminate the different treatments from each other. However, some general patterns could be highlighted. At 4h, the dispersion of the 4 replicates was higher for the algae treated by aldehydes, than those elicited or controls, suggesting more variability in metabolic profiles. At 4h, 8h and 28h, the

aldehyde-treated samples were clearly separated from the control points, even if some were close to each other or with the elicited samples, suggesting distinct metabolic profiles in elicited and aldehydes-treated algae compared to controls at each sampling time (Fig 3).

### **Aldehydes triggered production of FFAs and their oxidized derivatives**

Among the ten most discriminant ions between different treatments at each sampling time, five ions were selected, because annotated as putative FFAs or their oxidized derivatives. Three of these *in silico* annotations were confirmed by further LC-MS/MS analyses, i.e. the stearidonic acid (m/z 275.2 rt 603 sec), a jasmonic acid (JA) derivative compound (raw formula of C<sub>12</sub>H<sub>18</sub>O<sub>3</sub>, m/z 209.12 rt 364 sec) and a putative eicosapentaenoic acid (m/z 301.22 rt 679 sec). However, the two other ions (m/z 351.22 rt 346 sec and m/z 309.2 rt 403 sec) supposed to belong to oxylipins were not confirmed to be (13)-HOTrE and 20-hydroxy-leukotrien B4.

Figure 4 presents the relative amount of these 5 ions in *L. digitata*, along the time-course, according to the different treatments. Globally, 4-hour incubation in 4-HNE and 4-HHE led to a strong increase of their production 4 hours after incubation. This rapid induction was less pronounced for the dodecadienal and elicitation treatments, lasted up to 8h for the 4-HHE treatment, and was followed by a relative decrease for the 4-HNE and dodecadienal treatments. After 28h, the algae submitted to the dodecadienal treatment showed a stronger production peak for these compounds. More specifically, all the samples treated with the 3 aldehydes contained two to over four times more of the unknown compound m/z 351.22 compared to a control kelp at 4 hours, whereas its relative amount tended to be down-regulated after 8 or 28 hours. The stearidonic acid was rapidly more produced after 4-hour incubation in 4-HNE and 4-HHE than without treatment, and after 8 hours following the dodecadienal treatment. After 28 hours, the relative amount of this FFA was similar to that of the control algae, unlike in elicited algae where it reached twice more than the control level. For the 4-HHE treatment, a higher production of JA derivative was observed after 8 hours compared to other treatments. The relative amount of the unknown ion m/z 309.2 was similar or slightly increased in aldehyde-treated algae compared to control algae after 4h. However after 28h, its relative amount and that of the eicosapentaenoic acid were, respectively, 3 and 3.5 times more important after incubation in dodecadienal than after no incubation or after elicitation.

## **Discussion**

### **Aldehyde perception rapidly affected algal metabolism through different regulation pathways**

Here, we demonstrated that 4-HNE, 4-HHE and dodecadienal, applied at 100 mg.mL<sup>-1</sup>, did not induce an oxidative burst, as observed using oligoguluronate elicitors in *L. digitata*. Thus, we could hypothesize that the NADPH oxidase is not activated in response to these compounds and that they do



not act as exogenous elicitors like oligoguluronates (Küpper et al., 2001). This also suggests that these C6-, C9- and C12-aldehydes did not trigger strong oxidative responses as the level of H<sub>2</sub>O<sub>2</sub> in surrounding seawater was similar between aldehyde-treated and control algae. Reactive oxygen species (ROS) have already been shown to play roles in algal defense against pathogens. In red algae, ROS emitted were shown to have an antibacterial effect on *Gracilaria conferta* epiflora after oligogalacturonate elicitation (Weinberger and Friedlander, 2000). In *L. digitata*, the oxidative response is also an essential element of natural resistance against bacterial degraders and the oligoguluronate-triggered oxidative burst was shown to significantly increase resistance against infection by endophytic pathogens (Küpper et al., 2002). Altogether our results suggest that the 4-HNE-induced protection against herbivores do not involve the NADPH-activation pathway nor ROS emission.

Following incubation with 4-HNE, 4-HHE and dodecadienal, we have investigated the changes of the algal endometabolome during a time-course up to one day. The comparison of metabolic profiles based on 177 detected ions did not allow to strongly discriminate the different treatments (incubation in aldehydes, control or elicitation) whatever the sampling time. However, in the PLS-DA the metabolic profiles of the control algae were not grouped with those of aldehyde-treated neither with those of elicited *L. digitata*, at 4, 8 and 28 hours. For elicitation, it has already been shown that GG application specifically induced metabolic regulation, as shown by the massive release of halogenated volatile compounds and aldehydes in the medium surrounding *L. digitata* one hour after elicitation with GGs (Thomas et al., 2011). Similarly, the differences observed in metabolic profiles of the aldehyde-treated algae are likely to correspond to different metabolic regulations. All the three aldehydes tested are therefore perceived as signals by *L. digitata* and seem to be integrated to induce production of metabolites at different timeframe depending on their chemical nature.

We could follow the profiles of some compounds that were already known as induced in response to abiotic and biotic stresses (Ritter et al., 2008; Goulitquer et al., 2009). Interestingly, both aldehydes 4-HNE and 4-HHE strongly induced an early production of the 5 annotated compounds compared with control and elicited algae after 4h or 8h after treatment, whereas the dodecadienal's incubation showed their increases later (28h after algal treatment). The chemical nature of the annotated ions has been validated by LC-MS/MS analyses for stearidonic acid and a jasmonic acid derivative, suggesting the activation of FFA and oxylipin pathway by these three aldehydes. These results are consistent with the study of Bouarab et al. (2004) which demonstrated that elicitation with MAMPs or methyl jasmonate activated the metabolism of C20 and C18 PUFAs in the red alga *C. crispus*, generating prostaglandins and jasmonates. Similarly, Küpper et al. (2006) found that MAMPs and MIMPs (LPS and GG respectively) were shown to induce an increased release of C18 and C20 FFAs in *L. digitata* within 30 min. However, unlike LPS, GG did not induce a significant accumulation of some oxidized derivatives of linolenic and eicosapentaenoic acid after one hour, suggesting that oligoguluronate perception would activate restricted oxylipin biosynthetic pathways. Interestingly, the aldehyde 4-HHE applied on *L.*

*digitata* at the concentration of 1  $\mu\text{g}\cdot\text{mL}^{-1}$  was already shown to significantly increase the production of the oxylipin 13-(HOTrE) after 24 hours (Goulitquer et al., 2009). Moreover, the aldehyde dodecadienal induced a stronger release of stearidonic acid compared to other aldehydes. This fatty acid was already shown to be strongly produced in the red alga *C. crispus* in response to infection by the endophyte *A. operculata*, concomitantly with arachidonic acid and linolenic acid (Bouarab et al., 2004). Altogether our results suggest that 4-HHE, 4-HNE and dodecadienal could trigger the production of their own precursors by activation of different metabolic pathways.

### **Aldehydes showed antagonist effects on algal consumption according to their chemical nature**

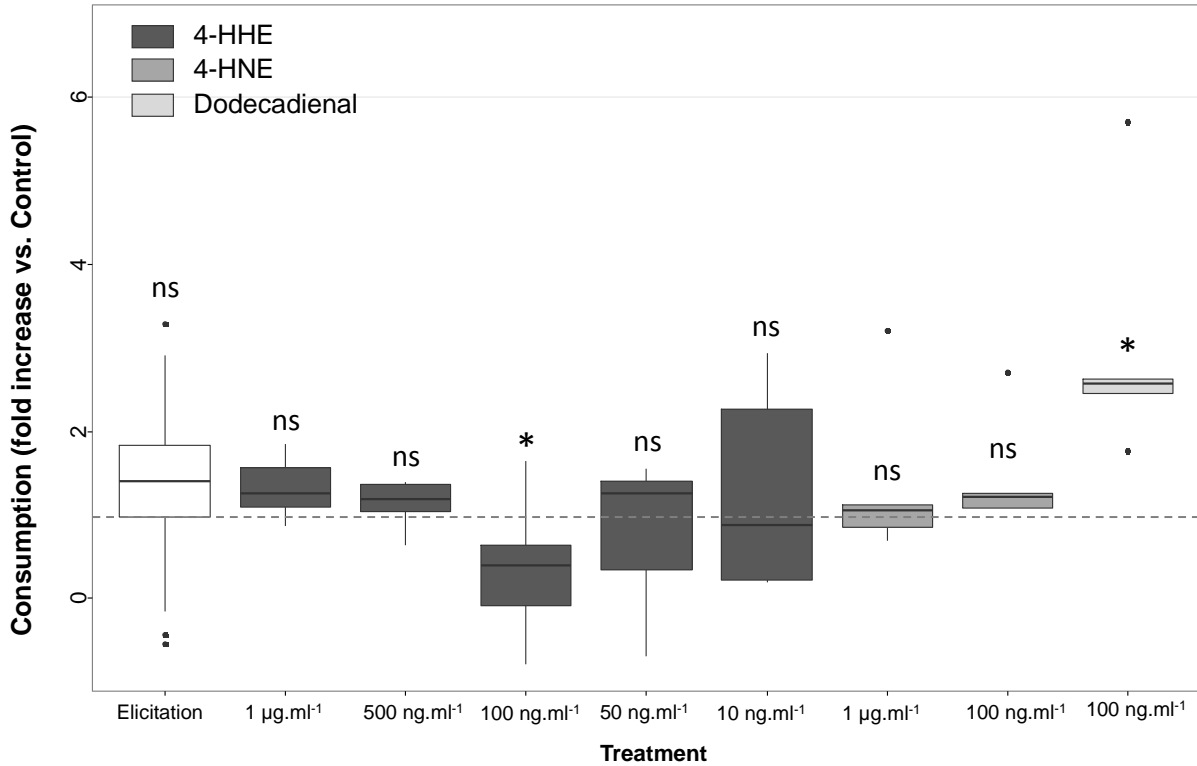
According to the nature of the aldehyde, *L. digitata* responded differently to a subsequent grazing by *P. pellucida*. Dodecadienal increased significantly the consumption by herbivores while 4-HHE decreased it when applied at 100  $\text{ng}\cdot\text{mL}^{-1}$ , and had no effect. The metabolic regulation observed in response to the aldehyde incubation suggested that dodecadienal, 4-HNE and 4-HHE were perceived by the kelp, and they seemed to induce different metabolic pathways in the kelp. If 4-HNE did not trigger protective responses towards herbivores, it could be not sufficient to activate defense responses against grazers. The different effect of 4-HNE and 4-HHE could also be due to the chemical properties of the compounds, such as solubility. Indeed, challenging of *L. digitata* with oligogulonates or copper stress showed a strong release of 4-HHE mostly in the water phase, while 4-HNE was rapidly transferred in the air phase (Goulitquer et al., 2009), related to a lower amount in the water phase. Dodecadienal was released in an equivalent way between the two phases (Goulitquer et al., 2009), but showed an antagonist effect than 4-HHE on algal consumption by grazers. This should be linked to the activation of particular metabolic pathways, as illustrated by the specific induction patterns of the five selected metabolite markers.

The optimal concentration of aldehydes for observation of algal protection was 100  $\text{ng}\cdot\text{mL}^{-1}$  of 4-HHE and 4-HNE. However, this concentration is substantially higher than aldehyde amounts that are released in the environment or by *L. digitata* following stress in laboratory experiments. Indeed, measured concentrations after 1h elicitation in the laboratory was around 0.2  $\text{ng}\cdot\text{mL}^{-1}$  4-HHE, 2.0  $\text{ng}\cdot\text{mL}^{-1}$  4-HNE and 4.0  $\text{ng}\cdot\text{mL}^{-1}$  dodecadienal. In the field, the highest concentrations of aldehydes measured over kelp beds were around 0.015  $\text{ng}\cdot\text{mL}^{-1}$  4-HHE 1h after low tide and 0.03  $\text{ng}\cdot\text{mL}^{-1}$  4-HNE 30 min after low tide (Goulitquer et al., 2009). This high difference in aldehyde concentrations could be explained, for instance, as these aldehydes may be emitted by numerous kelps in the same area, then perceived by the exposed neighbors, leading to efficient algal defenses against herbivores. We can also hypothesize that these compounds could be included in a blend of volatiles, involving a wide range of chemical compounds which could act synergistically on algal responses against grazing. The ratio of concentrations between the different compounds in the blend could also be crucial for the perception of the warning signal by neighboring kelps.

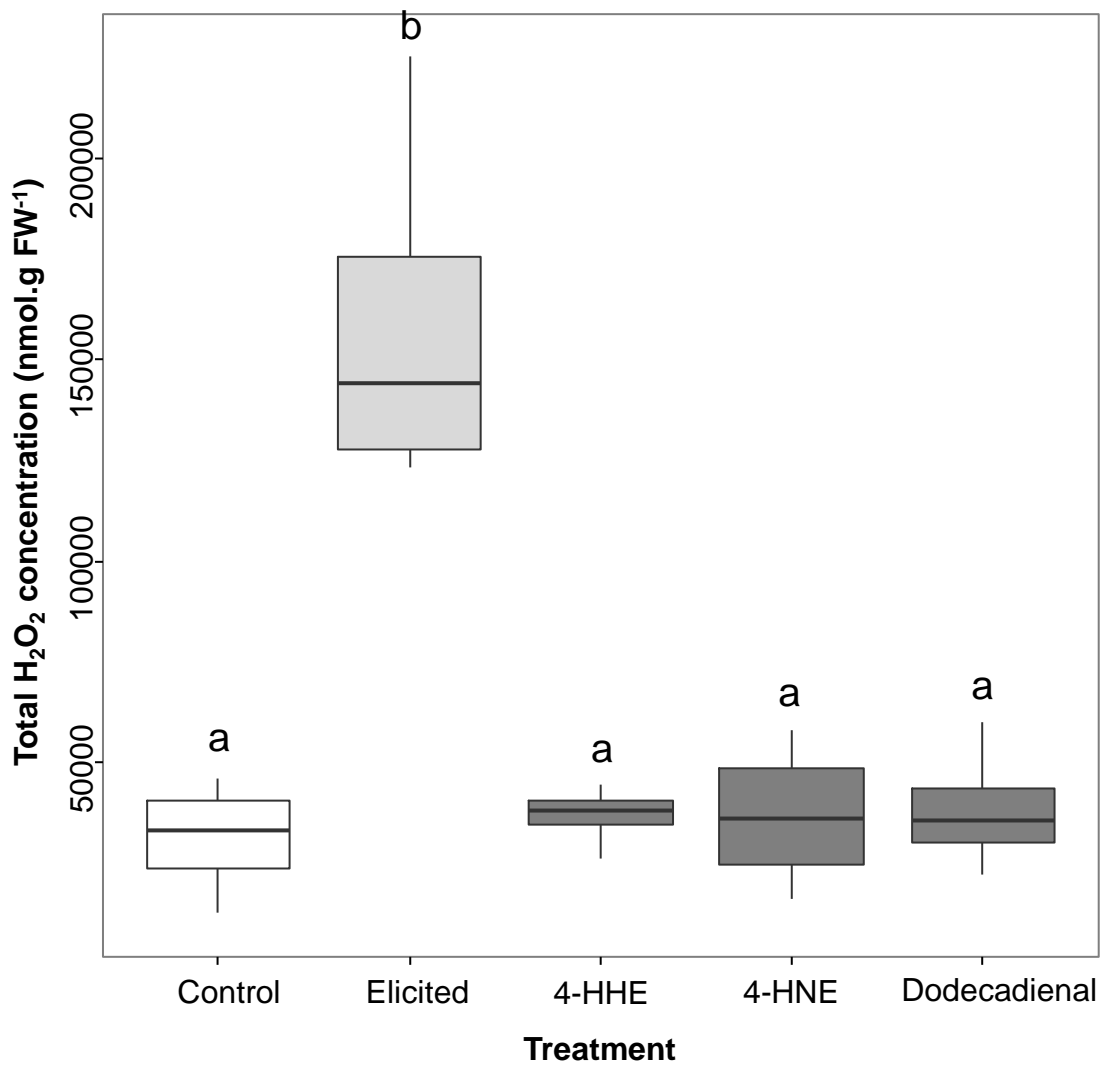
## Conclusion

In *L. digitata*, the incubation in different aldehydes modified algal consumption by herbivores and algal chemical profiles, suggesting their involvement as defense signals during kelp/herbivore interactions. Interestingly, according to their chemical nature, aldehyde signals have antagonist effects on protection against grazers, and seem to induce different metabolic pathways. For instance 4-HHE increased the resistance of the kelp within 1 day against the specialist herbivore *P. pellucida* and induced early production of fatty acids and oxidized derivatives after aldehyde application. At the contrary dodecadienal appeared to favor algal consumption, and a later induction of fatty acid metabolism. Further studies should be conducted to decipher the molecular mechanisms induced by these different aldehydes and evaluate their effects on generalist herbivores. This could lead to a better understanding of the aldehyde-based signals as defense chemical cues in the marine environment.

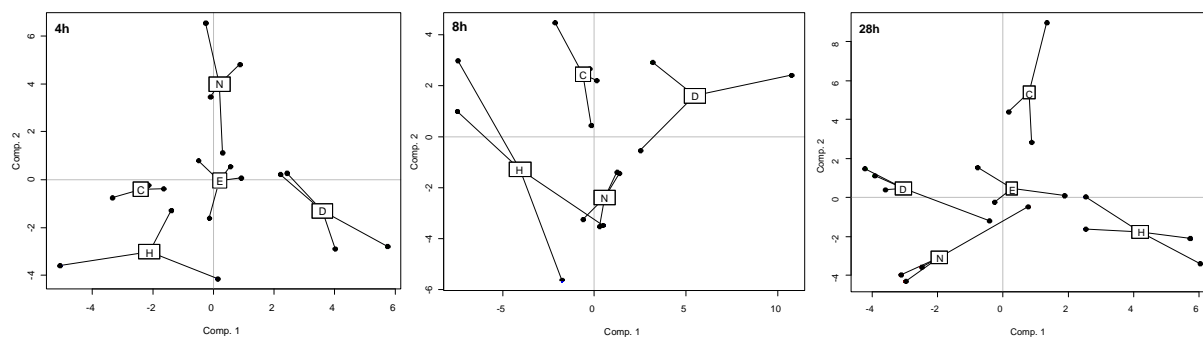
## Figure legends



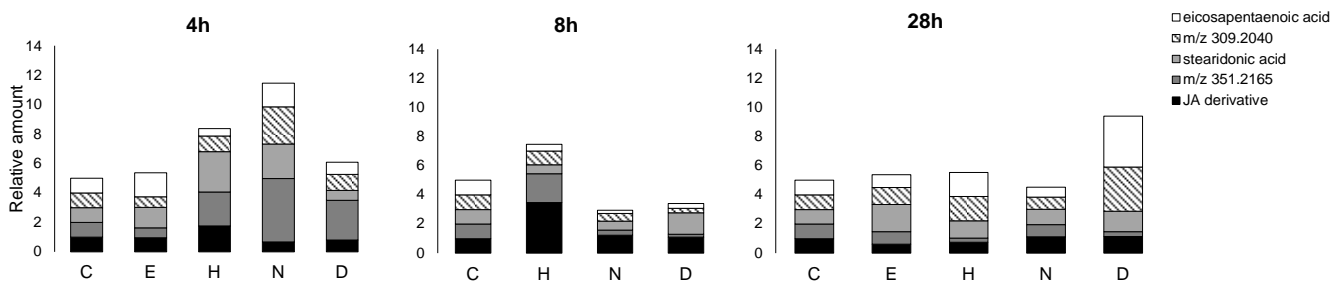
**Fig 1.** Effects of different treatments on consumption of *L. digitata* by *P. pellucida* grazers after 3 days. One day before putting in contact with herbivores, the algae were incubated either with GG for 1h (Elicited) or with different aldehydes for 4h, i.e. 4-HHE, 4-HNE or dodecadienal. Results are expressed in algal consumption (measured by fresh mass differences) relative to control (without treatments of algae). Elicited: 150 µg.mL<sup>-1</sup> (n=25). Aldehydes were applied on algae at the following concentrations: 4-HHE 1 µg.ml<sup>-1</sup> (n=5), 500 ng.ml<sup>-1</sup> (n=5), 100 ng.ml<sup>-1</sup> (n=15), 50 ng.ml<sup>-1</sup> (n=5) and 10 ng.ml<sup>-1</sup> (n=10); 4-HNE 1 µg.ml<sup>-1</sup> (n=5) and 100 ng.ml<sup>-1</sup> (n=5); dodecadienal 100 ng.ml<sup>-1</sup> (n=5); Wilcoxon tests were performed for each treatment, to compare the consumption of treated algae with the consumption of the control algae (without treatment). \* significantly different from the control; ns: non significantly different from the control.



**Fig 2.** H<sub>2</sub>O<sub>2</sub> production over 60 minutes in algal surrounding seawater after application of oligoguluronates at 150 µg.ml<sup>-1</sup> or of 4-HHE, 4-HNE or dodecadienal at 100 ng.ml<sup>-1</sup>. After checking normality, ANOVA analysis was performed: F-value = 19.976, p-value = 7.165e-06, SNK test: C=H=N=D<E (n=4). Letters above the error bars indicate groups that are significantly different (P < 0.05).



**Fig 3.** PLS-DA of the metabolic profiling based on 177 ions, from the endo-metabolome of *L. digitata* sampled at 4h, 8h and 28h after incubation in aldehydes 4-HHE (H), 4-HNE (N) or dodecadienal (D). Aldehyde treatments were compared with a control without treatment (C) and an elicited alga (E). MVA test, 999 permutations 4h: NMC = 0.69, p-value = 0.18; 8h: NMC = 0.82, p-value = 0.76; 28h: NMC = 0.78, p-value = 0.51.



**Figure 4.** Relative amount of five compounds in *L. digitata* sampled after 4h, 8h and 28h of incubation in aldehydes 4-HHE (H), 4-HNE (N) or dodecadienal (D). Results are compared with a control without treatment (C) and an elicited alga (E). For each compound, the control level was set to a relative unit of 1 to express the fold-variation in the other conditions (absolute concentration values are provided in Tables S1). Three compounds were identified by LC-MS/MS analyses as stearidonic acid (m/z 275.2 rt 603 sec), jasmonic acid derivative (raw formula of  $C_{12}H_{18}O_3$ , m/z 209.12 rt 364 sec), putative eicosapentaenoic acid (m/z 301.22 rt 679 sec).

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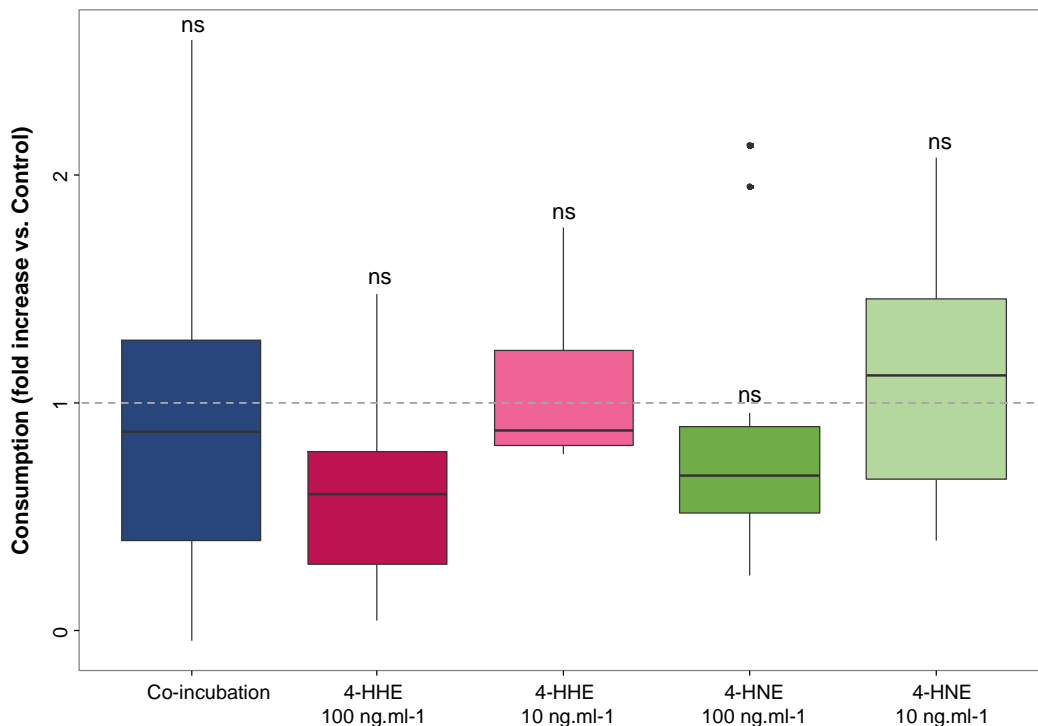
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## II. Aldehyde-based signaling in the interaction between *L. spicata* and the generalist herbivore *T. atra*

The results of this experiment were obtained by Y. Yacine in Las Cruces. Algal consumption of *L. spicata* was measured after 1 day and 2 days of grazing by *T. atra*, subsequently to treatment with either the aldehyde 4-HHE or the 4-HNE, or after a co-incubation with grazed *L. spicata* fronds. Two independent experiments were performed using application of aldehydes at 100 ng.mL<sup>-1</sup>, and consumption of control kelps was not significantly different between the two experiments after 1 day of grazing (Wilcoxon test, W=28, p-value = 0.72) and after 2 days (W=38, p-value = 0.57). Results of these experiments were thus grouped in a single dataset. Fig. 22 shows the results of algal consumption after 1 day of grazing after the different treatments (algal consumption after 2 days of grazing is not presented as the same results were obtained). Incubation of *L. spicata* in the 4-HHE and in the 4-HNE at 10 ng.mL<sup>-1</sup> did not induce any tendency of algal consumption by grazers, whereas treatment with the aldehydes at 100 ng.mL<sup>-1</sup> tends to decrease the consumption, although not significantly. Co-incubation with grazed kelps, considered as a reference for distance signaling treatment, did not induce any modifications of algal consumption in comparison to control algae.



**Figure 22.** Ratios of algal consumption after one day by *T. atra* of treated *L. spicata* either with 4-hydroxyhexenal (4-HHE) or 4-hydroxynonenal (4-HNE) or co-incubated with a grazed kelp (co-incubation) over control algae (without treatment). ns indicates no significant difference between the treated kelps and the control (Wilcoxon test,  $\alpha=0.05$ ). n=8.

## Discussion

### 1. Aldehydes have antagonist effects on algal protection against herbivores

Investigations on the effects of aldehydes on algal consumption by both generalist and specialist herbivores showed that kelps seemed to respond differently according to the chemical nature and concentration of the aldehyde. Whereas no effect was observed with 4-HNE, *L. digitata* consumption by *P. pellucida* was significantly reduced after application of 100 ng.mL<sup>-1</sup> 4-HHE, but significantly increased after application of dodecadienal. In the kelp *L. spicata*, the tendencies were similar with the decrease of the consumption by *T. atra* after an application of 4-HHE and 4-HNE on the algae compared with control algae without treatment. Secondly, the aldehyde concentration applied on the kelp appeared to influence the effect on a subsequent grazing. Indeed, only the 100 ng.mL<sup>-1</sup> concentration of 4-HHE applied on *L. digitata* significantly decreased algal consumption, while lower and higher concentrations did not seem to have any effect on *P. pellucida*'s feeding behavior. Similarly, the concentration of 100 ng.mL<sup>-1</sup> was effective to decrease consumption of *L. spicata* with both aldehydes 4-HHE and 4-HNE. Thus, these results obtained in two distant kelp species suggest that 4-HHE could play a common role in aldehyde signaling and defense against generalist and specialist herbivores. 4-HNE could also be involved in similar defense processes, suggesting that they could both act on the same metabolic pathways. On the contrary, dodecadienal that was shown to favor the consumption by herbivores is likely to induce other metabolic pathways. Altogether these results suggest that as in plants and diatoms, aldehyde-based signaling occurs in kelps and could have a role during interactions with herbivores. Specific aldehydes are likely to interfere with the regulation of distinct metabolic pathways, leading to antagonist physiological responses, such as protection or attraction of herbivores.

### 2. Aldehydes are inducers of algal metabolic regulations

Studies on the metabolism changes in algal tissues in response to the three aldehydes showed that these compounds did not induce an oxidative burst, suggesting that they act downstream the activation of the NADPH oxidase. Indeed, the metabolic profiles of *L. digitata* were clearly modified after 4-hour incubation with aldehydes, compared to control. This supports the hypothesis of both perception and biological integration of the aldehydes by the algae as chemical signals. More specifically, we showed that they triggered the production of polyunsaturated fatty acids and oxylipins, with different timeframes according to the aldehyde: 4-HHE and 4-HNE induced a rapid increase (between 4 and 8h after incubation in aldehydes), while dodecadienal triggered it later (1 day after incubation). Thus, the increased algal consumption after dodecadienal application could be explained by an induction or a repression of some metabolic pathways, for instance involved in production of fatty acids and their derivatives, in contrast to 4-HNE and 4-HHE which could stimulate defense pathways. However, other

chemical compounds could act on algal defenses, constituting a complex blends in which some compounds such as 4-HHE and 4-HNE would act synergistically for a better defense of the kelp.

### 3. Could aldehyde-based signaling be efficient in the natural environment?

The optimal concentration of aldehydes for observation of effects on algal protection was  $100 \text{ ng.mL}^{-1}$  for 4-HHE and 4-HNE. However, this concentration is substantially higher than aldehyde amounts that found in the environment and previously measured during laboratory experiments. Indeed, measured concentrations after 1h elicitation in the laboratory was around  $0.2 \text{ ng.mL}^{-1}$  4-HHE,  $2.0 \text{ ng.mL}^{-1}$  4-HNE and  $4.0 \text{ ng.mL}^{-1}$  dodecadienal. In the field, the highest concentrations of aldehydes measured over kelp beds were around  $0.015 \text{ ng.mL}^{-1}$  4-HHE 1h after low tide and  $0.03 \text{ ng.mL}^{-1}$  4-HNE 30 min after low tide (Goulitquer et al., 2009). In our experiments, the lowest concentration ( $10 \text{ ng.mL}^{-1}$ ) did not show any effect. This high difference (around 1 to 5000 times more) between aldehyde concentrations *in situ* and that showing a biological effect on kelp protection in the laboratory could be explained, for instance, if these aldehydes would be emitted by several kelps in a same area, highly increasing local concentrations, that could be perceived by neighbors, leading to efficient algal defenses against herbivores. We can also hypothesize that these compounds could be part of a blend of volatiles, involving a wide range of chemical compounds likely including other aldehydes, which could act synergistically on algal responses against grazing. In the light of our results, this complex blend could include the aldehyde 4-HHE for a better protection of *L. digitata* against *P. pellucida*. In addition to the nature of the chemical compounds, the ratio of concentrations between the different compounds in the blend could also be crucial for the perception of the warning signal by neighboring kelps and in efficient chemical signaling during defenses against herbivores.



# **Conclusions and perspectives**

## 1. Two complementary approaches in chemical ecology: metabolomics and bio-assays

Metabolic profiling approaches are powerful global methods for comparing metabolic states of algae submitted to different treatments over time. These methods allowed to highlight global metabolic regulations that were set up in kelps in response not only to direct grazing stress but also to indirect grazing stress. We found that kelps regulated their inner metabolism locally in the wounding area and after co-incubation with a grazed neighbor, as well as their exo-metabolome, after perception of exogenous elicitors. The metabolic regulations included changes in their relative amounts in fatty acids and their oxidized derivatives, and in their amino acid content during the first two days. The transient modification in fatty acid algal content could also be linked to the observed herbivore attraction and could participate to the perception of a remote food source, although other chemical cues could be involved in attraction behavior of herbivores. Moreover, these endo- and exo-metabolome varied over time, at least within two days. More specifically, we chose to target compounds deriving from the fatty acid pathway, hypothesizing that this metabolism could be involved in a potential distant communication inside and outside algal tissues, as already suggested by several previous studies in the laboratory. A metabolomic global approach could present some limits at various levels. The data acquisition and their quality depend strongly on the initial quality of the biological samples, which is highly determined by the extraction method. Indeed, in order to get a view as large as possible of a metabolome, we usually performed extraction techniques using the mix MeOH/H<sub>2</sub>O which allowed an extraction of a wide range of compounds. However, this often caused troubles during metabolomic analyses, as too many ions were detected and could thus hide the interesting information, i.e. the differentially-induced compounds, explaining physiological modifications. Optimizations of the extraction method as well as of the analytical techniques are thus crucial for chromatograms and mass spectra of reasonable qualities. Moreover, data processing was performed using the Workflow4metabolomics platform, which is a bioinformatic tool, thus still under evolution, comprising more and more precise functions for a better adequacy to the data. This has to be taken into account by regularly updating functions to optimize the analysis. Beyond the global metabolomic analyses, it is worthwhile to underline that the final annotation of ions, is often a bottleneck, as the available chemical databases still contain a limited number of marine chemical compounds, especially from algae. Most of actual knowledge is still sparse and mainly available through previous studies or through collaborations with other labs working in marine research domain.

The complementary approach of metabolomics in chemical ecology is the setting up of bio-assays for testing the biological or ecological role of identified chemical compounds. Thanks to non-choice and multiple choice bio-assays developed and carried out during this thesis project, we first showed that recently grazed kelps were more attractive and subsequently more consumed by generalist and specialist herbivores than control kelps without treatments and their co-incubated neighbors. However, some global conclusions could not be drawn, as numerous uncontrolled factors interfered with our

observations. For instance, we showed that the season had a strong effect on algal physiology and on its consumption by grazers. The experimental setups have thus to be improved in order to take into account this side effect, for example by conducting several bioassays in parallel at the same period of the year. The experimental design of co-incubation of two kelps could be performed using a grazed frond in a box upstream to a naïve frond, which will only receive the water flow from the grazed alga. This would avoid the negative stress effect of the net on the grazed kelp and its reciprocal effect on its neighbor. These external factors are even stronger in *in situ* experiments, influenced for instance by the effects of the waves, reducing the relevance of the results. Increasing the number of replicates thus plays a central role in relevant bio-assay experiments, due to the various hardly controllable external factors.

Combining metabolic profiling and ecological approaches, another prospect of this research could focus on a bio-guided fractionation of the soluble chemical compounds emitted by a kelp having a strong effect on protection against grazing. Successive filtration of the complex blend of emitted compounds associated with bio-assays of their effects on algal consumption could lead to the identification of the chemical families acting on kelp/herbivore interactions.

## 2. Grazed kelps seem to better attract grazers and be more consumed

Unexpectedly, we observed that challenging kelps by grazing for 2 days or defense elicitation by oligoguluronates generally stimulated a subsequent grazing by herbivores and also attracted them. In *L. spicata*, we showed that, only in summer, the kelp could gain weight and be less consumed when it was previously grazed by conspecific herbivores. This season effect was interpreted as the occurrence of upwelling on Chilean coasts in this season (between September and January) which could provide nutrients to the water surface, leading to higher algal growth and potentially better defenses against aggressors. On the contrary, during the rest of the year, the kelps could be less provided in nutrients, thus containing less energy for growth and defense. However, the effect of a previous grazing on subsequent algal consumption was once observed after a previous grazing, but, in that case, not after elicitation by oligoguluronates. Kelps could therefore be more attractive for herbivores only after grazing. This could suggest that a natural grazing by herbivores and a simulated grazing by oligoguluronate would induce different defense mechanisms in kelp tissues upon a subsequent grazing, implying activation or repression of different metabolic pathways according to the treatment (grazing or elicitation). In *L. digitata*, specialist as well as generalist herbivores seem to consume preferentially kelps for which defenses were elicited 24 hours before, leading to regulations of metabolism. In the case of *L. spicata*, in some conditions, the grazing stress over two days is likely to induce the release of specific attractive compounds at the surface of the thallus or in seawater, while chemical elicitation did not. Therefore, kelps have the capacity to actively respond to herbivore damage, but the actual triggers and metabolic pathways activated during this process might differ among species/season/ecosystem. It can be



hypothesized that herbivores could perceive distance compounds coming from these challenged algae, detected as degradation products of the kelp, and thus as an edible food source. Interestingly, this observation has been made in the two kelp species, suggesting that this attraction trait could be common in kelps, either inherited from the common ancestor, or originating from evolutionary convergence. The direct effects of fatty acids and oxylipins on feeding by herbivores could be tested through incorporation of pure or mixed compounds in artificial food, given to generalist and specialist grazers. For further investigating the attraction of herbivores in a natural environment, *in situ* experiments could also be performed on *L. digitata* in order to test the natural recolonization of grazers in a calm environment compared with a medium constantly under influence of waves.

Moreover, it seemed that this attraction depended on the relationship between the algal host and the herbivore. Preliminary experiments showed that the generalist grazers *T. atra* were attracted to the kelp *L. spicata* when it was previously grazed by their conspecifics, while they could not discriminate control algae from kelps previously grazed by the specialist herbivore *S. scurra*. This may reflect a kind of recognition of the interaction between the kelp and the herbivore to following grazers, like the food source could be recognized as edible only after grazing by the same species. We can suggest that this could be a strategy for herbivores' fitness, which could identify a remote food source, thus avoiding poisoning or unnecessary movement towards non-edible diet.

### 3. Kelps perceive and integrate water-borne signals emitted by their grazed neighbors

When a kelp was co-incubated with a grazed one, metabolic regulations occurred in both algal tissues but these changes were clearly different between the two treatments. However, the co-incubation with a grazed kelp activated different metabolic regulations than co-incubation with an unchallenged alga. Thus, the grazed kelp released chemical signals in the surrounding seawater that could induce specific modifications in the receiving kelp. The different metabolic pathways induced in response to the perception of chemical compounds released in the seawater upon a grazing stress could lead to adaptive responses to a subsequent herbivore attack. Interestingly, in both *L. digitata* and *L. spicata*, field observations showed high concentrations of *P. pellucida* and *S. scurra*, respectively, on a single kelp, while its neighboring conspecifics contained limited numbers of grazers on their thalli. Given the low dispersion rate of kelp spores in the marine environment, it is admitted that close neighbors have family relationship. From the evolutionary theory of the kin selection, we suppose that the over-grazed kelp should favor the development of its offspring around, possibly through the emission of warning signals and/or the attraction of grazers, thus increasing the global fitness of the algal species. Even if chemical signaling could require metabolic costs for its production and release, it could therefore give advantages to the species, by providing benefits to the descendants.

#### 4. Aldehydes are inducers of metabolic responses and modify algal resistance to herbivores

At the wounding site, some released compounds following grazing by herbivores and the endometabolome of a co-incubated kelp with a grazed alga were shown to be fatty acids and oxidized derivatives oxylipins. Elicitation by oligogulonates and copper stress were already shown to induce the release of a wide range of aldehydes in both the air and the seawater (Ritter et al., 2008; Goultquer et al., 2009). This previous study demonstrated that the aldehyde 4-HHE induced the synthesis of an oxylipin, and proposed that some aldehydes could act as inducers of metabolic responses. Our results suggested that the metabolic oxylipin pathway could also be involved in kelp responses to grazing stresses. Aldehydes seem to induce the production of their own precursors (oxylipins), forming an induction loop which could then be involved in switching on warning mechanisms towards the neighboring kelps for an optimized defense against grazers.

The direct effects of aldehydes on algal defense against herbivores, evaluated through algal consumption bioassays, seem to strongly depend on the chemical nature of the aldehyde, as well as on the concentration applied on the kelp. For instance, 4-HHE and 4-HNE seemed to decrease the grazing pressure in both kelp species treated by  $100 \text{ ng.mL}^{-1}$ , whereas dodecadienal appeared to stimulate algal consumption at this concentration. In parallel, metabolic profiling showed that the algal endometabolome was rapidly modified after application of these aldehydes, in particular regarding fatty acids and oxylipin amounts. These modifications were observed early in time after algal incubation in 4-HHE and 4-HNE (4 to 8 hours), while occurring later after incubation in dodecadienal (around 24 hours). All these results strongly suggest that the three aldehydes induced different metabolic pathways in kelp tissues, some being involved in algal defense responses.

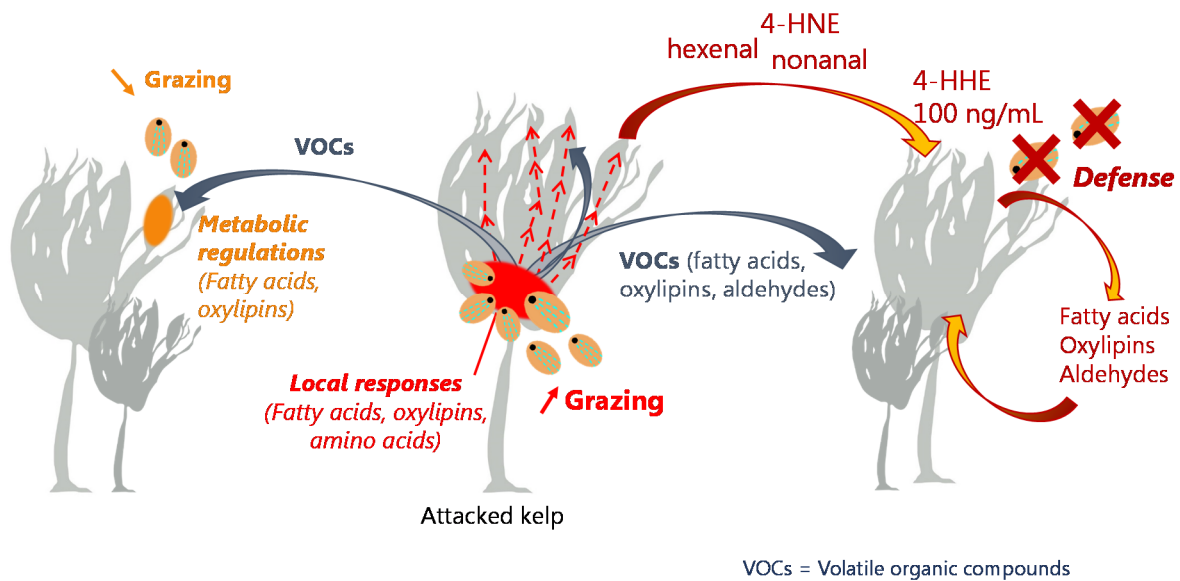
Some aldehydes, such as 4-HHE, could be perceived by kelps in the closely neighborhood of the grazed kelp, then inducing a release of free fatty acids, their oxidation, thus forming *de novo* aldehyde production. This should result in an amplification of metabolic responses to grazing, to finally inhibit kelp grazing by either specialist or generalist herbivores. However, numerous compounds should be involved in this chemical signaling, acting altogether synergistically to activate an optimized defense against the herbivore. In contrast to 4-HHE, the aldehyde dodecadienal increased the consumption by grazers, suggesting that this compound could be perceived differently from 4-HHE and could induce pathways that do not trigger defenses of the kelp against grazers.

Moreover, the fact that these aldehydes are emitted in much lower amounts in natural conditions than the efficient concentrations in laboratory experiments could suggest that a single chemical compound would not be sufficient for triggering of defenses. Indeed, the signal could contain a complex blend of chemical compounds, acting together on algal defense induction and later protection against herbivores. Rather than being composed of specific compounds which could require high costs for their production, this bouquet could contain relative ratios of compounds constitutively present in kelps which could

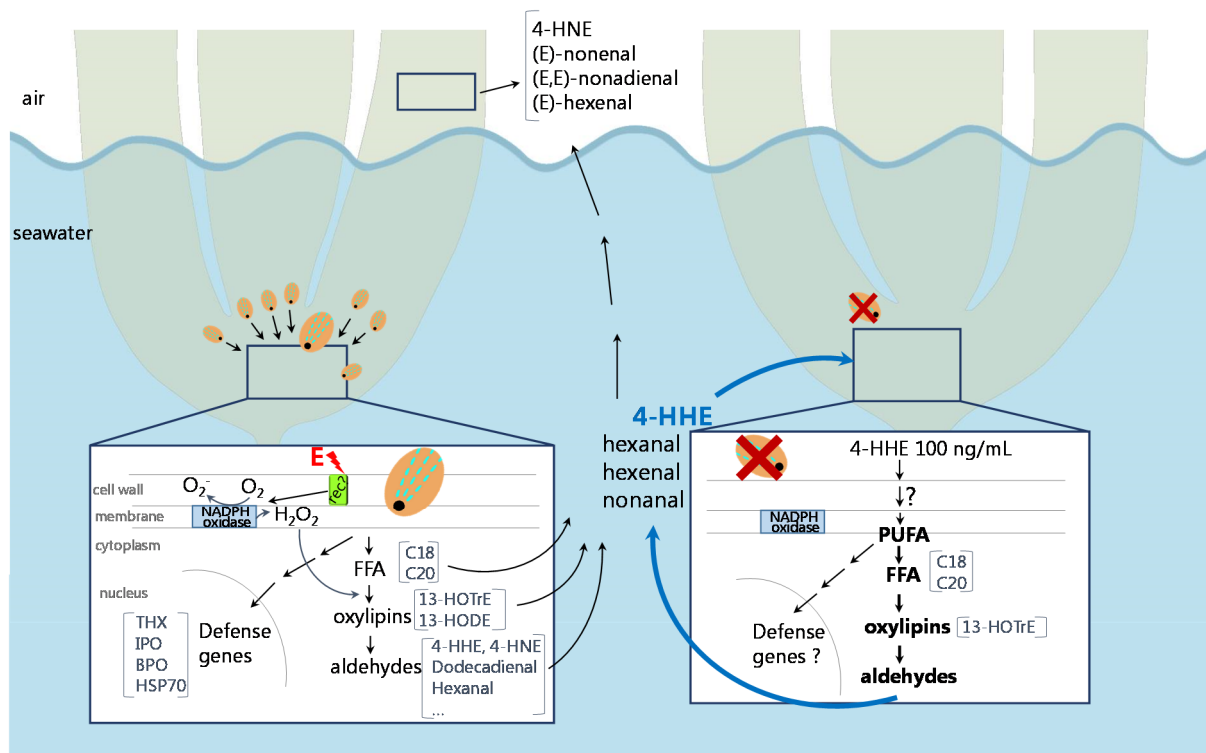
induce algal defense. Moreover, it can be supposed that, in the natural environment, several kelps could respond to grazing stress, thus increasing the amounts of the chemical compounds into the seawater and allowing their perception by neighboring kelps, leading to defenses. The fact that 4-HHE was perceived by kelps, without inducing an oxidative burst, but triggering fatty acid metabolism regulation and defense responses against *P. pellucida* could suggest that this aldehyde could induce defenses in neighboring kelps during kelp/herbivore interactions.

This study should be completed by further bio-assays testing the effects of other aldehydes and oxylipins which were found in kelps in response to diverse stresses in natural conditions or in the laboratory, on algal consumption by grazers. These compounds could be tested alone and in mixture as well, using different ratios between the chemical compounds. It could also be interesting to evaluate their effects on the interaction between kelps and generalist herbivores, as well as their direct effect on grazers at different concentrations, through artificial food experiments.

In conclusion, this work allow to draw new general schemes regarding chemical signaling in kelps during interactions with herbivores (Fig 23 and 24). We highlighted the existence of chemical compounds linked to fatty acid metabolism released during grazing, such as aldehydes, and their biological role on metabolism of neighboring algae and on their defense against grazers. Like in plants and marine diatoms, aldehyde-based signals seem to play a crucial role in regulation of kelp defense mechanisms. These are the first components to continue the deciphering of fine chemical interactions between kelps and their associated herbivores, and of underlying mechanisms.



**Figure 23.** Schematic representation of distance signaling in kelp chemical defense during interactions with herbivores. In response to grazing by herbivores, local responses are activated such as the accumulation of fatty acids, oxylipins and amino acids, and grazers are firstly attracted to the wounding site. Simultaneously, the kelp releases diverse Volatile Organic Compounds (VOCs) into the surrounding environment, like fatty acids, oxylipins and aldehydes. Some of these emitted compounds have impacts on metabolic regulations in neighboring kelps, activating the fatty acid metabolism in algal tissues. Among the emitted compounds, the 4-HHE is perceived by the neighboring kelp, changing its metabolism and then inhibiting further grazing by herbivores.



**Figure 24.** Schematic representation of biochemical processes in kelp chemical defense in response to grazing. Grazing by herbivores induces a release of free fatty acids which are oxidized by ROS, producing oxylipins and aldehydes, as well as activation and repression of defense genes. Some chemical compounds are emitted into the seawater and a part of them reach the air environment. Among these compounds, the aldehyde 4-HHE is perceived by the neighboring kelp and induces modification of metabolism, leading to protection against herbivores.

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# Appendices



## **Appendix 1.**

Daily Rhythm of Mutualistic Pollinator Activity and Scent Emission in *Ficus septica*: Ecological Differentiation between Co-Occurring Pollinators and Potential Consequences for Chemical Communication and Facilitation of Host Speciation

**Authors:** Lucie Conchou<sup>1\*</sup>, Léa Cabioch<sup>1</sup>, Lillian J. V. Rodriguez<sup>1,2</sup>, Finn Kjellberg<sup>1</sup>

**Affiliations:**

<sup>1</sup> CEFE UMR 5175, CNRS - Université de Montpellier - Université Paul-Valéry Montpellier – EPHE, Montpellier, France,

<sup>2</sup> Institute of Biology, University of the Philippines Diliman, Quezon City, Philippines

**Corresponding author:** lucie.conchou@gmail.com

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# Daily Rhythm of Mutualistic Pollinator Activity and Scent Emission in *Ficus septica*: Ecological Differentiation between Co-Occurring Pollinators and Potential Consequences for Chemical Communication and Facilitation of Host Speciation

Lucie Conchou<sup>1\*</sup>, Léa Cabioch<sup>1</sup>, Lillian J. V. Rodriguez<sup>1,2</sup>, Finn Kjellberg<sup>1</sup>

<sup>1</sup> CEFE UMR 5175, CNRS - Université de Montpellier - Université Paul-Valéry Montpellier – EPHE, Montpellier, France, <sup>2</sup> Institute of Biology, University of the Philippines Diliman, Quezon City, Philippines

## Abstract

The mutualistic interaction between *Ficus* and their pollinating agaonid wasps constitutes an extreme example of plant-insect co-diversification. Most *Ficus* species are locally associated with a single specific agaonid wasp species. Specificity is ensured by each fig species emitting a distinctive attractive scent. However, cases of widespread coexistence of two agaonid wasp species on the same *Ficus* species are documented. Here we document the coexistence of two agaonid wasp species in *Ficus septica*: one yellow-colored and one black-colored. Our results suggest that their coexistence is facilitated by divergent ecological traits. The black species is longer-lived (a few more hours) and is hence active until later in the afternoon. Some traits of the yellow species must compensate for this advantage for their coexistence to be stable. In addition, we show that the composition of the scent emitted by receptive figs changes between sunrise and noon. The two species may therefore be exposed to somewhat different ranges of receptive fig scent composition and may consequently diverge in the way they perceive and/or respond to scents. Whether such situations may lead to host plant speciation is an open question.

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\* Email: lucie.conchou@gmail.com

## Introduction

Plant-insect interactions are at the origin of huge diversification within the living world [1]. Plants need to attract pollinators and defend themselves against phytophagous insects, resulting in chemical diversification, and this diversity has led to radiations of whole insect groups. Despite the biological importance of this diversity, how plants and their associated insect communities diversify is largely unknown. For instance, what levels of ecological specialization will accelerate or impede the macroevolutionary diversification process is still largely unknown [2].

The mutualistic interaction between *Ficus* and agaonid wasps constitutes an extreme example of plant-insect co-diversification [3]. Agaonid wasps only develop in figs (the urn-shaped inflorescence of *Ficus*) of their host species (generally one host species, sometimes more [4]), and they are almost the sole pollinators of *Ficus* [5]. While many of the 800+ currently recognized *Ficus* species seem to be associated with a single pollinator species, some have two or more [6]. In some exceptional cases, co-pollinators of a *Ficus* species belong to different genera, a

feature which should facilitate co-existence through strong ecological differentiation, as when diurnal *Elisabethiella* species coexist with nocturnal *Alfonsiella* species [7]. Much more often, the pollinators associated with a given *Ficus* species are sister species. The presence in different parts of the range of a *Ficus* species of different but closely related pollinator species could be an intermediate step in a process of allopatric speciation in which pollinator speciation could precede and maybe facilitate host speciation. In such situations, two or more species of pollinators may be observed to co-occur locally, but only in contact zones between their respective ranges. Examples include the three species of the *Wiebesia pumilae* complex associated with *Ficus pumila*, [8], and the two species associated to *Ficus sur* in West Africa. In the latter case, *Ceratosolen flabellatus* is a forest specialist, *C. silvestrianus* is a savanna specialist and both species coexist in forest-savanna mosaics [9].

In some situations, however, two or more sister species of pollinating wasps colonize the same trees and often even the same figs, over larger parts of the range of a *Ficus* species. In such

situations, we may expect strong interspecific competition between the wasp species that should select for niche differentiation. For instance, co-occurring sister species could evolve different compromises between dispersal capacity (which in fig wasps is tightly linked to lifespan as these very short lived wasps disperse by drifting in the wind) and competitiveness (e.g. the ability to rapidly locate receptive figs, to enter them, and to oviposit faster than the other pollinator species).

There are five documented cases of relatively widespread co-occurrence of closely related pollinator species on a fig host, for which habitat differentiation has not been suggested as the mechanism allowing co-existence. The first example concerns *Ficus microcarpa* in Hainan [10] but no biological information or wasp description are provided and the evidence for widespread co-occurrence is preliminary. In Yunnan, however, one of the two sister species of agaonid wasps colonizing *F. microcarpa* does not carry pollen so that its larvae develop mainly in unfertilized fig ovules as opposed to mainly in fertilized ones in the case of pollinators [11], suggesting that, in this case, co-occurring sister species present highly divergent ecologies [12]. The second example concerns the co-occurrence of the sister species *Elisabethiella stuckenbergi* and *E. socotrensis* on *Ficus natalensis* in South Africa [4]. *E. stuckenbergi* has a shorter head than *E. socotrensis*, a characteristic that suggests different fig-entering capacities since head shape of agaonid wasps is strongly correlated with the shape of the entrance into the fig [13].

The three other examples involve differences in coloration among otherwise morphologically-similar wasp species. *Ficus tuerckheimii* is pollinated by two co-occurring species of *Pegoscapus* in both Mexico and Costa Rica [14]. *Pegoscapus carlosi* is uniformly black while *Pegoscapus mariae* is ventrally honey-colored and dorsally blackish [15]. Similarly, in Australia, *Ficus rubiginosa* is pollinated by four sister species constituting the *Pleistodontes imperialis* species complex [16]. The color varies from entirely dark testaceous (all specimens collected close to the city of Sydney, New South Wales), to dorsally testaceous and ventrally yellow (some specimens collected close to the city of Mareeba, Queensland), to nearly completely yellow (some specimens collected close to the city of Townsville, Queensland) [17]. The yellow-colored individuals correspond to one cytotype while the correspondence between color and cytotype is not yet ascertained for the other color morphs. Finally, *Ficus septica* is pollinated by a yellow and a black species that co-occur in south Taiwan [18], a situation we have also observed in populations from the Philippines. In both *F. septica* and *F. rubiginosa*, only the dark colored pollinators are present at the more temperate limits of their distributions.

In fig-pollinating wasps, light body color (qualified by authors as yellow, amber or honey) is almost always associated with large eyes and nocturnal flight, while dark color is always associated with smaller eyes and diurnal dispersal. In the three aforementioned cases however, both dark and light sister species have relatively small and similarly-sized eyes that suggest diurnal flight [15,17,19,20]. In insects, melanisation or lack of melanisation may correlate with a diversity of adaptive traits [21]. Production of melanin is physiologically costly in insects: it can only be maintained if it confers a selective advantage [22]. For instance intra-population color variation in *Drosophila melanogaster* correlates strongly with resistance to dehydration, with light morphotypes being most sensitive [23]. The three cases reported above constitute the sole examples we have detected, in a systematic survey of pollinating fig wasp descriptions, of most probably diurnal light colored fig pollinating wasps. Therefore, we predict that the light color corresponds to an unusual life history

strategy in diurnal pollinating fig wasps that is made possible because of selection for ecological niche separation between closely related species using the same resources. Given classical explanations of advantages associated with dark color, we surmise that dark pollinating wasps are better protected against diurnal stresses such as oxidative stress due to exposition to UV and ozone. We therefore predict that differences in body color might correlate with trade-offs between competitiveness and lifespan. Diurnal fig-pollinating wasps usually emerge from their natal fig early in the morning and survive only a few hours [24–26]. The black species, being better protected, would survive longer and have a more extended fig colonization time window than the yellow species. As a consequence it would be sufficiently long lived to reach somewhat more isolated receptive figs. Under the competitiveness-lifespan trade-off scenario, the yellow species would be more efficient than the black one at rapidly locating and entering receptive figs but would be shorter lived. A similar trade-off was found in the dark and pale males of the non-pollinating *Walkerella* sp. associated to *Ficus benjamina*, where males fight to access females [27]. Pale males were better fighters whereas dark males were more susceptible to disperse out of their natal figs in search for alternative mates. Dark males also tended to survive longer in laboratory conditions.

In *Ficus*, scent is the major cue used by pollinators to locate receptive figs and it facilitates the species-specificity of the interaction: each *Ficus* species produces a specific volatile blend that is only attractive to its specific pollinators [28–31]. However, variation of this volatile blend during the course of a day is expected as it has been reported for flowers as well as leaves of other plant species [32–34], and potential consequences of such variation on fig wasp behavior has not been investigated. One source of this variation originates in the plants being exposed, as the wasps, to a variety of stresses during daytime, involving oxidative stresses and thermal stresses. Volatile isoprenoids, including monoterpenes, protect plant tissues against these stresses [35,36], and their emission varies during the day [37]. Monoterpenes are also perceived by insects and are involved in their attraction to plants (e.g. in fig wasps [38,39]). We may therefore expect daily variation of the scents produced by receptive figs to result from responses to selection stemming from both daily patterns of pollinator activity and daily variation in abiotic stress intensity. On the contrary, leaves do not contribute to pollinator attraction [40] but they are exposed to the same abiotic stresses as figs. Daily leaf scent variation can therefore be used as a control to disentangle both functions in figs, and determine whether daily fig scent variation is the result of an adaptation to increase pollinator attraction. Moreover, the presence of two pollinators instead of one is an exceptional situation for a fig species, and this may have selected for particular adaptations. It is therefore necessary to include a second type of control: a closely related *Ficus* species pollinated by a single wasp species.

Whatever the adaptive reasons are behind daily fig scent variation, we may expect adaptation of the pollinators to recognize the range of receptive fig scents to which they are regularly exposed. Two pollinator species presenting different daily activity patterns could be exposed to somewhat different ranges of attractive scent composition. If the hypothesis of more extended daily activity period of the black pollinators is upheld, and if scents emitted by receptive figs vary during the day, then we may predict some divergence over evolutionary times in the way the black and the yellow wasp species perceive and respond to volatile cues, a trait that could ultimately facilitate host plant speciation via assortative mating among plant genotypes if there is some heritable variation in receptive fig scent composition.

In this contribution, we document pollinator coexistence in *Ficus septica*. 1) We show that the yellow and black pollinator species associated with *Ficus septica* emerge from figs in the morning but differ in their lifespan, 2) we compare the daily rhythm of scent production by receptive figs with the rhythm of scent production by leaves and by figs and leaves of the closely-related *Ficus nota* and 3) we document that the composition of *Ficus septica* receptive fig scent varies during the course of a day. We discuss the potential consequences of these findings for the future evolution of this system, especially in terms of diversification processes.

## Materials and Methods

### Biology of the model system, study site and species

The fig is an urn-shaped inflorescence. Its inside is lined by uniovulate female flowers and male flowers. When the fig is receptive it emits a scent that attracts pollinating wasps. The wasps enter the fig, oviposit in some of the female flowers and pollinate. Female wasps that have colonized a fig and deposited their offspring in it are called foundresses. In monoecious *Ficus* species, seeds and galled flowers that each contains a single wasp larva develop side by side. Some weeks later, male wasp offspring emerge into the fig cavity and mate with the females still enclosed in their galls. The female wasps then emerge into the fig cavity, become loaded with pollen, and leave in search of a new, receptive fig. Finally, the fig ripens, becoming attractive to a large set of frugivorous animals. In dioecious *Ficus* species, functionally-male trees bear figs that produce wasps, pollen, but no seeds. Female trees bear figs that do not allow wasp oviposition and do not produce pollen, but do produce seeds. The adult lifespan of pollinating fig wasps is usually a few hours and is entirely devoted to searching for a receptive fig and subsequently ovipositing inside that fig. In *Ficus* in general, and in *Ficus septica* in particular, flowering is relatively synchronous within a given tree but asynchronous among trees resulting at the population level in the production of receptive figs and adult wasps throughout the year. The set of figs developing synchronously on a fig tree is called a crop.

The experimental work was carried out in the Diliman Campus of the University of the Philippines, in Quezon City, on the island of Luzon, Philippines (N 14°38'E 121°03'). Because the work was carried out in the campus of the University, no specific permit was required to conduct this study. Further, none of the studied species is protected or endangered. The campus is located in an urban zone and a large part of its area is made of more-or-less natural patches of vegetation. In the campus, native *Ficus* species grow wild in gardens, along roads and streams and in less intensively-tended places. Experimental work was carried out during the dry season, from January 14<sup>th</sup> 2013 to April 12<sup>th</sup> 2013. During this period, sunrise shifted from 6:30 a.m. to 5:45 a.m. and sunset from 5:45 p.m. to 6:15 p.m.

In Quezon City and more generally in the island of Luzon, *F. septica* is associated with two closely-related *Ceratosolen* species belonging to the *Ceratosolen bisulcatus* species complex (F. Kjellberg, L.J.V. Rodriguez unpublished observations, J.Y. Rasplus pers. com.): *Ceratosolen jucundus*, yellow-colored [19], and *Ceratosolen* sp., black-colored. Hereafter, they will be called “yellow pollinator” and “black pollinator” respectively.

In order to determine if the presence of two pollinators could have induced a shift in the period of the day when *Ficus septica* is pollinated and in the daily rhythm of fig scent production, some parallel observations were also done on a *Ficus* species associated to a single pollinator species. *Ficus nota* is taxonomically close to

*F. septica* (both belong to subgenus *Sycomorus*, section *Sycocarpus*, subsection *Sycocarpus*) and is pollinated by the black-colored *Ceratosolen notus*, a close relative of the pollinators of *F. septica* [3]. Both species are found throughout the Philippines. A set of male trees of both species was surveyed every 2–3 days for the presence of figs and their developmental stage. Trees bearing figs close to receptivity or close to wasp emergence were visited daily.

### Traits associated with wasp lifespan and competitiveness

The aims of the following experiments were 1) to examine differences between black and yellow pollinators in traits associated with their daily activity patterns and 2) to test whether the daily patterns of yellow and black pollinator emergence and presence around receptive *Ficus septica* trees differ from those of the pollinator species associated to *Ficus nota*.

**Wasp emergence patterns and lifespan of the black and yellow pollinators of *F. septica*.** At 4–6 p.m., we collected male *F. septica* and *F. nota* figs from which wasps were to emerge on the following day. These figs were recognizable due to their swelling and their softness. Each fig was put into a separate plastic pot closed with plankton net. The figs were then stored outdoors under the shade of a tree so that physicochemical conditions were as close as possible to *in natura*. Wasp emergences from figs were recorded every hour on the following day, from 5 a.m. to sunset.

During the survey of wasp emergences, pots containing *F. septica* figs from which both black and yellow pollinators were emerging were put aside. The fig was removed from the pot in order to keep only the insects that had emerged during the preceding single hour. Dead pollinators were counted after 6 hours and every 3 hours thereafter until all the insects were dead. The survey was replicated on eight figs taken from five different mother trees on six different dates. Therefore, yellow and black species survival rates were first compared for each fig separately.

For every fig, all the insects were dead within 12 hours after their emergence. We therefore calculated for each fig the survival rate of emerging black and yellow pollinators at two time points: six and nine hours after emergence. To determine if one species had a longer lifespan than the other, we performed a binomial test for each time point: we ranked yellow and black species survival rate and transformed this ranking into a binomial variable  $B$  ( $B = 0$  if yellow survival > black,  $B = 1$  if black survival > yellow). Under the null hypothesis (yellow and black lifespan identical) we expected to observe  $B = 1$  with a probability of 0.5 i.e. black survival rate to exceed yellow survival rate for just half of the figs. Our prediction was that black wasps would survive longer than yellow wasps ( $p(B = 1) > 0.5$ ).

### Day round pattern of pollinating wasp presence on trees during their period of fig receptivity.

In order to determine the day round presence on trees bearing receptive figs of the two pollinator species associated with *F. septica* and of the pollinator associated to *F. nota*, passive insect traps were hung in male trees bearing receptive figs. The insect traps were made of transparent A4 plastic sheets, rolled into a cylinder and coated with transparent odorless glue. Four such traps were suspended in branches close to receptive figs. The first insect traps were installed at 6 a.m., and were replaced every three hours until 6 a.m. on the following day. The experimental day was thus partitioned into eight time intervals. The exact timing of first trap setup, transition between fourth and fifth trap as well as last trap removal were adapted to match sunrise and sunset as close as possible. The experiment was repeated on six *F. septica* and three *F. nota* trees. After trap collection, the number of pollinators of each species captured during each three-hour time interval was counted.

Our prediction was that numbers of wasp trapped would decrease as the day progressed 1) because of limited lifespan of the wasps emerging in the morning and/or 2) because all the day's supply of receptive figs would no longer be attractive as they would have been pollinated in the morning. The following experiment was set up to discriminate between these two hypotheses.

**Consequences of manipulating the period of accessibility of receptive *F. septica* figs on the abundance of black and yellow foundresses.** Two branches bearing figs close to receptivity on each of three *F. septica* male trees were enclosed in plankton net bags for four to five days in order to let the figs become receptive without being pollinated. On one of the two branches per tree, the bag was removed at sunset the day before the experiment, at a time when pollinators were no longer active. Figs on this branch were thus accessible to pollinators for the whole experimental day. On the second branch the bag was removed at 0:30–1 p.m. on the day of the experiment, so that figs were accessible to pollinators only during the afternoon. All the figs were collected at sunset on the day of the experiment, and the number of black and yellow wasps that had penetrated each fig (number of foundresses) was determined. For each pollinator species, the mean number of foundresses per fig was compared between figs exposed the whole day and those exposed only in the afternoon, using Student's *t*-tests.

Our prediction was that because a lower proportion of the yellow pollinators would be alive and visiting figs in the afternoon than in the morning, figs exposed only during the afternoon would contain a higher proportion of black foundresses than fig already exposed to pollinators in the morning.

#### Daily pattern of scent production by *F. septica* and *F. nota* at the time of fig receptivity

The aims of the following experiment were to establish 1) whether volatile organic compound (VOC) release by figs varied in quantity and/or in composition during the course of a day, 2) whether the pattern observed in figs is similar to the pattern observed in leaves or whether it is adjusted to the daily pattern of pollinator activity, and 3) whether *Ficus septica* figs display an unusual pattern (potential adaptive response to the presence of two pollinators using *Ficus nota* figs as a control).

**Scent sampling design.** We compared daytime variation in volatile emissions (sunrise and noon) between receptive male figs of *Ficus septica* (two pollinators) and *Ficus nota* (one pollinator) and between figs and leaves.

Pre-receptive figs were enclosed in plankton net bags in order to prevent pollination. Large numbers of pollinators flying around the tree was taken as a signal that many figs had become receptive. We then performed receptive fig scent and leaf scent extractions simultaneously, once at sunrise and then once at noon. This sampling protocol was repeated in five male individuals of each *Ficus* species.

Flowering phenology was somewhat asynchronous in both *Ficus* species, so that it was necessary to select the figs from which VOC emissions were collected in order to obtain a sample as homogenous as possible in developmental stage. To avoid any bias due to haphazard allocation of figs to sunrise and noon extractions, we randomized the selection process. Prior to each sunrise scent collection, 40 figs were chosen from the tree to be sampled and 20 of them were randomly assigned to the sunrise scent collection. The remaining 20 were used for the noon scent collection.

**Scent extraction methods.** Scent extraction was performed using the headspace technique [31]. The filters were designed to fit inside a chromatoprobe thermodesorption kit (see next section)

and filled with 1.5 mg of Carbotrap 20–40 and 1.5 mg of Tenax 60–80. One microliter of a solution of internal standards (nonane and dodecane) in known concentrations was injected in each filter prior to extraction to allow later estimation of emission rates. Each sample was taken from either 20 receptive figs (selected as indicated above) or five leaves, cut off from branches and enclosed in polyethylene terephthalate bags. We standardized bag size to limit the variability in headspace volume and improve the repeatability of emission rate estimation. Scent was left to accumulate inside the bags for 30 minutes, and then the air was pulled out of the bag through the filter for five minutes with a flow rate of 160 mL/min. For each paired sample (one fig sample and one leaf sample extracted simultaneously), a control was made using an empty bag.

**Identification and quantification of the volatile compounds.** GC-MS analyses were carried out using a gas chromatograph CP-3800 (Varian Inc., Palo Alto, CA) equipped with an FID detector and coupled with a Saturn 2000 mass spectrometer (Varian). The samples were injected using a 1079 programmed temperature injector with a chromatoprobe kit (Varian), and was programmed as follow: 40°C hold for 0.5 min, and increased to 250°C at 200°C/min, hold for 3 min, and finally cooled down to 40°C with a fan. Chromatographic separation was performed using a fused silica capillary column (30 m×0.25 mm×0.25 μm Optima 5 Accent, Macherey-Nagel, Düren, Germany) with the following oven program: 40°C hold for 3 min, from 40°C to 100°C at 3.3°C/min, from 100 to 140°C at 2.9°C/min, from 140 to 180°C at 2.7°C/min, and finally upped to 250°C at 10°C/min and hold 8 min. The carrier gas was helium with a constant flow rate set close to 1.0 mL/min. The samples were injected in splitless mode. The energy for ionization by electron impact was 70 eV. The temperature of the transfer line, manifold and trap were respectively 250°C, 80°C and 170°C. The spectrometer was used in scan mode, from 38 to 300 *m/z* ratio.

All the volatile organic compounds (VOC) were tentatively identified by comparison with mass spectral libraries NIST98 MS and Adams 2007 [41], and retention indices found in Adams 2007 [41], online libraries (pherobase [42], NIST webbook [43]) and published data for (Z)- and (E)-DMNT [44]. Internal standards injected into each filter prior to scent extraction (0.08 μg nonane, 0.1 μg dodecane) allowed estimating the quantity (μg) of each identified compound contained in each sample.

**Statistical analysis of scent profiles.** Only VOCs that appeared in at least two different scent samples were retained to determine scent profiles. We checked that the presence/absence of the major VOCs was not affected by cutting off figs and leaves from branches. The only major VOC whose presence was due to cutting was (Z)-3-hexen-1-ol. Therefore, all the statistical analyses presented below were done after removing (Z)-3-hexen-1-ol from scent profiles. This had no qualitative effect on the results unless mentioned. From this VOC set, we calculated total emission rate and the relative composition of each scent profile. Total emission rates were the sum of emission rates of all VOCs detected in a given sample, calculated as μg/fig\*hour for figs and as μg/cm<sup>2</sup>\*hour for leaves. Relative scent composition was the relative contribution of each VOC to the scent profile, expressed as a percentage.

Emission rate variation among species and extraction hours were analyzed separately on figs and leaves, using Wilcoxon signed rank tests. Relative scent composition variation among species, organs, and extraction hours were analyzed with methods based on Bray-Curtis distances, implemented in the R package Vegan [45,46]: Patterns of variation were visualized using non-metric multidimensional scaling (NMDS, [31]) and their significance

tested with PERMANOVA [47]. NMDS is an ordination method which computes a locus for each sample within a space of given dimensionality so that the distances between samples on the final ordination are as close as possible to the original distances. The discrepancy between distances on the graph and actual distances is measured by the stress value, which varies from 0 (perfect correspondence) to 100% (no correspondence). According to the subset of samples to be included, we set the dimensionality to either 2 or 3 in order to always obtain stress values below 15%.

For the factors whose effect was detected to be significant by the PERMANOVA, we identified individual VOCs that contributed most to the overall difference by performing univariate Mann-Whitney tests between couples of sample categories. We tagged individual VOCs for which the p-value was lower than 0.05. We preferred this method to the dedicated test in the vegan package (simper function) because the latter is known to highlight variables presenting the highest intragroup variance rather than those that differ among groups [48]. Indeed, the simper function produced biologically meaningless results on our dataset.

## Results

### Traits associated with wasp lifespan and competitiveness

**Wasp emergence patterns and lifespan of the black and yellow pollinators of *F. septica*.** Most emergences from figs occurred in the morning in both *F. nota* and *F. septica*, independently of the pollinating wasp species. Indeed for 61% of 117 *F. septica* figs and for 57% of 89 *F. nota* figs, peak pollinator emergences occurred before 7 a.m. We did not detect any difference in timing of peak wasp emergence between *F. septica* figs hosting yellow, black or both pollinator species, and when both species emerged from the same fig they did so simultaneously. Since only 15 *F. septica* figs contained black pollinators (alone or together with yellow wasps), however, we could not exclude some slight difference in timing.

For all eight *Ficus septica* figs for which emerging pollinator lifespan was followed, all insects were dead within 12 hours after their emergence, so that survival was counted for each fig 6 hours and 9 hours after emergence. There was no single case of higher survival rate of yellow pollinators comparatively to black ones,

resulting in globally significantly higher survival of black wasps 6 and 9 hours after emergence (Table 1).

**Day round pattern of pollinating wasp presence on trees during their period of fig receptivity.** Black *F. septica* pollinators were much less abundant than yellow ones during the field session (1043 yellow wasps caught versus 71 black ones), a pattern also observed when we were monitoring emergences. Both *F. septica* pollinators and *F. nota* pollinators were virtually always caught during daytime on sticky traps (Figure 1, Table S1). In *F. septica*, the presence patterns of black and yellow wasps were very similar (Figure 1): most individuals were caught in the morning (87% of yellow and 85% of black pollinators were caught between sunrise and 12 a.m., see Table S1 for detailed results), with detections decreasing during the afternoon and approaching zero during the night. Given the difference in lifespan between both species, we would have expected the relative frequency of black wasps around receptive trees to peak in the afternoon. However, in addition to wasp lifespan, the actual presence of attractive figs must also influence the daily patterns of wasp presence around receptive trees. Morning-pollinated figs could rapidly lose attractiveness, a feature that could explain the low numbers of black wasps trapped on the trees in the afternoon. The following experiment was set up to test that hypothesis.

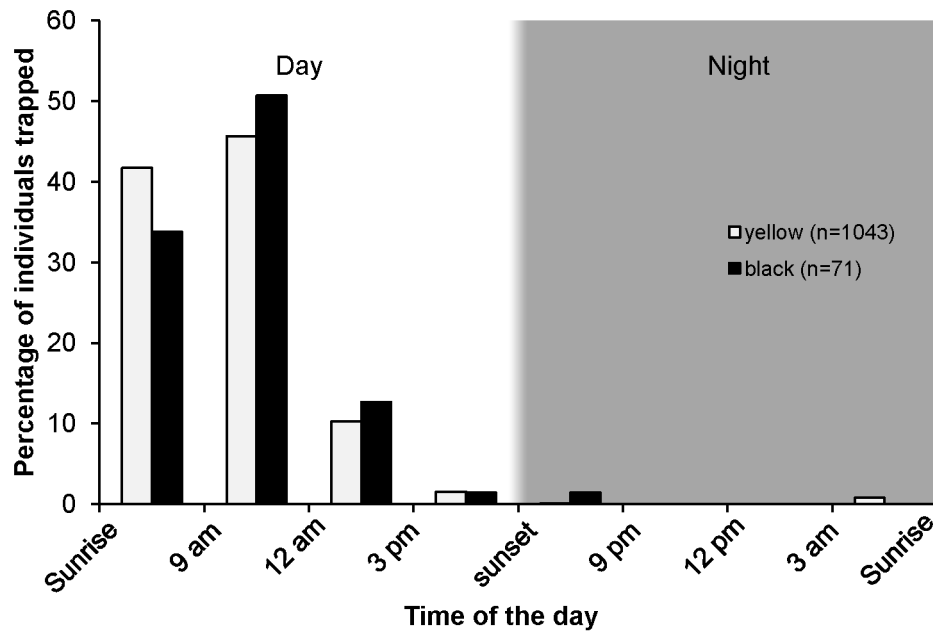
**Consequences of manipulating the timing of accessibility of *F. septica* figs on the abundance of black and yellow foundresses.** The figs that had been left accessible to wasps all day long contained many more yellow foundresses at the end of the experimental day than those that had been left accessible only during the afternoon (Wilcoxon rank sum test:  $W = 815$ ,  $p < 0.001$ , Figure 2). On the contrary, the number of black foundresses that had entered the figs did not differ according to their period of accessibility ( $W = 357.5$ ,  $p = 0.35$ , Figure 2). The mean proportion of black wasps was significantly higher in figs left accessible only during the afternoon (whole day:  $5+/-9\%$  black wasps; afternoon:  $34+/-28\%$  black wasps; generalized linear model with quasibinomial distribution:  $t = -3.43$ ,  $p = 0.0011$ ). An interpretation of these results is that decreased pollinator densities in the afternoon in natural conditions could be due to a rapid loss of fig attractiveness once pollinated. We propose that when some figs remained attractive in the afternoon, black pollinators were more efficient at colonizing them probably because of their longer

**Table 1.** *Ficus septica* pollinating wasp lifespan survey: compared survival rates of yellow and black pollinators emerged from the same figs.

fig N°	number of wasps		survival rate (%)			
			6 hours after emergence		9 hours after emergence	
	yellow	black	yellow	black	yellow	black
1	28	150	28.57	78.66	0	5.33
2	106	24	56.60	75.00	0	4.16
3	136	39	84.55	100.00	0	30.76
4	29	28	48.27	89.28	0	0
5	31	125	77.41	81.60	0	0
6	77	36	58.44	88.88	14.28	38.88
7	214	57	77.10	98.24	5.14	75.43
8	203	25	46.79	60.00	0	12.00
binomial test p-value <sup>1</sup>			0.0039		0.016	

<sup>1</sup>null hypothesis: if yellow and black pollinators have the same lifespan we expect that at any point in time the survival rate of the black species exceeds that of the yellow species in half of the replicates (p-values are for one-tailed tests, excluding ex-aequo).

doi:10.1371/journal.pone.0103581.t001



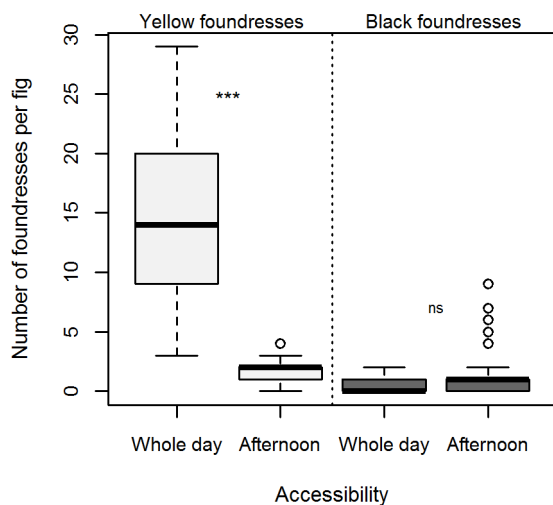
**Figure 1. The daily pattern of *Ficus septica* pollinator activity around trees bearing receptive figs.** Number of yellow and black pollinators trapped at different times of the day, expressed as percentage of the total number of individuals trapped over 24 hours on 6 different trees. Raw data provided as Table S1.

doi:10.1371/journal.pone.0103581.g001

lifespan. Because wasp lifespan should be counted in hours, the longer longevity of black pollinators should enable them to colonize more distant host trees.

#### Daily patterns of scent production by *F. septica* and *F. nota* at the time of fig receptivity

**Variation in scent emission rates.** Figs and leaves of the two species displayed the same pattern of variation: emission rates were significantly higher at noon than at sunrise (Wilcoxon signed rank test on both species pooled; figs:  $V = 8$ ,  $p = 4.8 \times 10^{-5}$ ,



**Figure 2. Colonization by pollinators of *Ficus septica* receptive figs whose accessibility has been manipulated.** Number of yellow (light grey) and black (dark grey) foundresses found inside figs that have been left accessible to pollination for the whole day or in the afternoon only. Raw data provided as Table S2.

doi:10.1371/journal.pone.0103581.g002

Figure 3.A.; leaves : Figure 3.B.,  $V = 8$ ,  $p = 0.049$ ). Hence the general physiology of the trees and protection against abiotic stress is sufficient to explain the rhythm of fig VOC production. We have no evidence in favor of an emission rhythm that would reflect adaptation to pollinator activity rhythm or number of pollinator species.

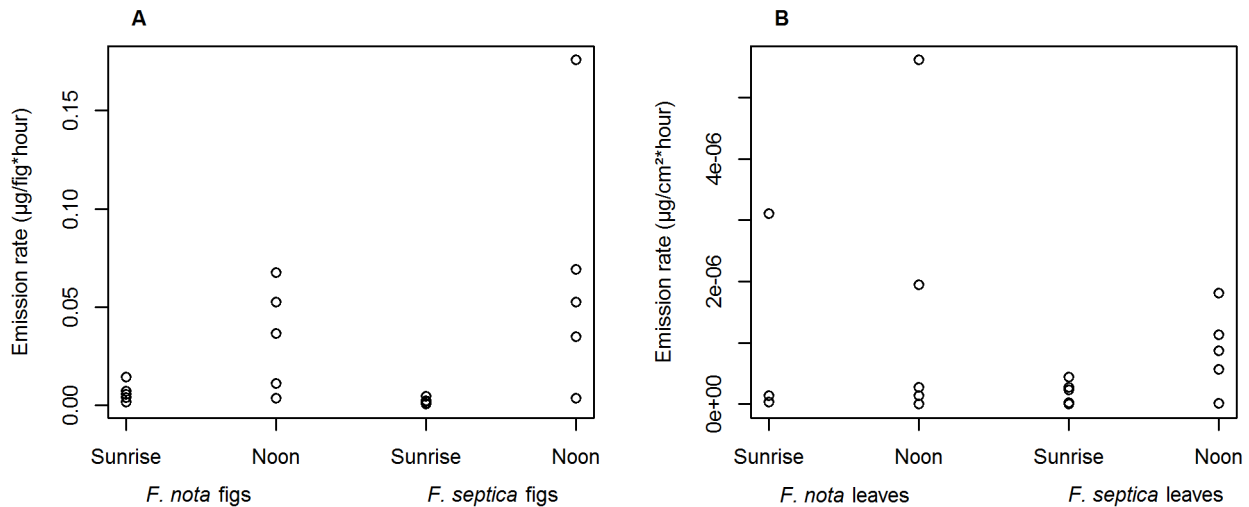
**Variation in relative scent composition.** Seventy VOCs were detected in at least two samples and retained in the computation of scent profiles (Table S3, all statistical analyses exclude (Z)-3-hexen-1-ol, see material and methods). Forty-eight of them were tentatively identified. A further 16 of the non-identified ones were assigned to a biosynthetic category. Both *F. septica* and *F. nota* emitted mainly terpenoids (mean relative contribution varying from 48 to 94% depending on the species, organ and hour of extraction). Overall, figs emitted a larger number of different VOCs than leaves, and noon scents were comprised of a larger number of VOCs than morning scents (Table S3).

Relative scent composition varied significantly according to species, organ type and time of extraction (global PERMANOVA, Table 2). The species\*organ type and organ type\*time of extraction interactions were also significant. On the 3 dimensional NMDS ordination, fig and leaf scents were separated along axis 1 (Figure 4). Morning and noon scents were separated on axes 2 (Figure 4.A) and 3 (Figure 4.B, Wilcoxon rank sum tests comparing position along the axes:  $W = 326$  and  $p = 0.0004$  for axis 2,  $W = 331$  and  $p = 0.0002$  for axis 3).

In order to get better insights into sources of variation, we performed some further analyses separately on fig and leaf scents.

**Separate analysis of daily variation in fig and leaf relative scent composition.** Sixty-seven VOCs were present in at least two fig samples. Receptive fig scent composition differed significantly between the two species and according to time of extraction (PERMANOVA, Table 3). The interaction term had no significant effect, suggesting that the two effects were orthogonal. Indeed, on the 2 dimensional NMDS ordination (Figure 5.A), the two species are separated along axis 1, and





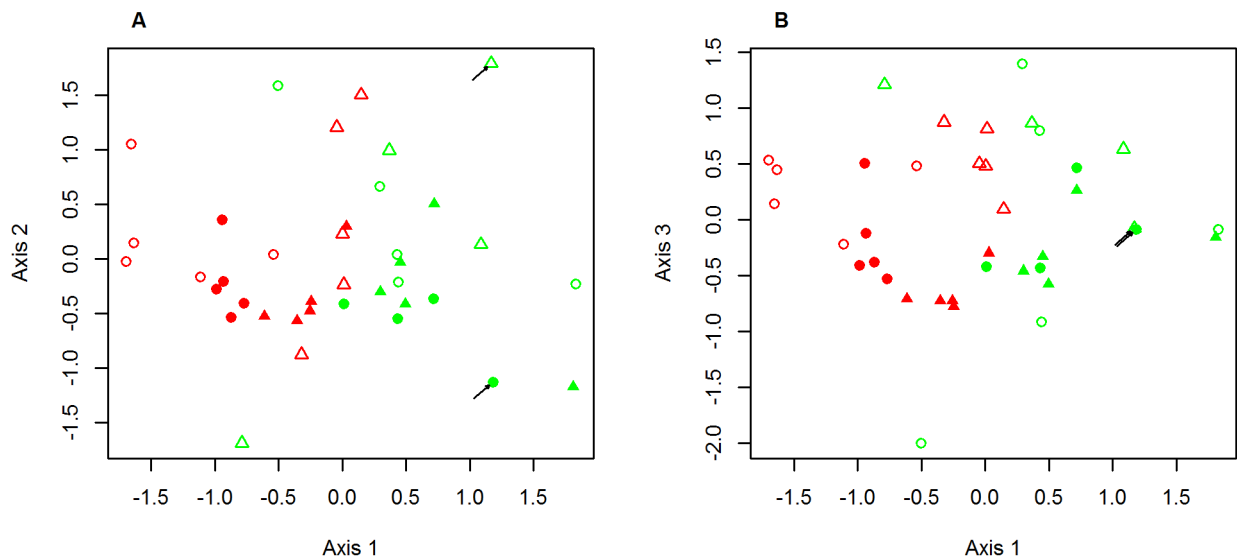
**Figure 3. Daily variation of scent emission rates in *Ficus septica* and *Ficus nota*.** Total scent emission rates from (A) figs ( $\mu\text{g}/\text{fig}\cdot\text{hour}$ ) and (B) leaves ( $\mu\text{g}/\text{cm}^2\cdot\text{hour}$ ) of both species at sunrise and at noon. doi:10.1371/journal.pone.0103581.g003

sunrise and noon samples along axis 2. Again, this suggests the absence of special features in *Ficus septica* scent production rhythm.

Twenty VOCs were present in at least two leaf samples. Leaf scent composition varied significantly according to the hour of extraction, but the species effect and the interaction term were not significant (PERMANOVA, Table 4). Although we observed no segregation on the NMDS ordination (Figure 5.B.), sunrise samples were more dispersed than noon samples. Sunrise leaf scent composition was actually poorly consistent across samples, as only one VOC ((E)-caryophyllene) was detected in at least 4 out of 5 sunrise leaf samples in *F. nota*, and none in *F. septica*. In other words, we cannot define a clear mean profile for leaf scents in the morning. (Z)-3-hexen-1-ol was the only other compound consis-

tently present in most morning leaf samples. When it was included in the analysis, sunrise and noon leaf scents were segregated along axis 1 of the NMDS ordination (not shown). The lack of consistency in the composition of morning leaf scents may be due to low emission rates, as most compounds detected in these samples were at or near to the detection limits.

**Identification of the VOCs responsible for the difference between sunrise and noon scents.** Regardless of species and organ type, the difference between sunrise and noon scent composition was mainly explained by a set of monoterpenes and both DMNT enantiomers (Table 5), whose relative proportion was higher at noon. The exact identity of the VOCs involved was different in figs of both species and in leaves, but (Z)- and (E)- $\beta$ -ocimene were common to all. Consistent with this, there was a



**Figure 4. Patterns of variation of the composition of scents emitted by *Ficus septica* and *Ficus nota*.** Three-dimensional NMDS ordination on the relative composition (% each VOC) of scents emitted by figs (red symbols) and leaves (green symbols) of *Ficus septica* (triangles) and *Ficus nota* (circles) at sunrise (open symbols) and at noon (closed symbols). (A) Axes 1 and 2, (B) Axes 1 and 3. Stress-value = 10%. Black arrows indicate places where two samples of the same category share the same locus. doi:10.1371/journal.pone.0103581.g004



**Table 2.** PERMANOVA analysis on the relative composition of scents emitted by *Ficus septica* and *Ficus nota* figs and leaves at sunrise and noon.

Factor	Df	sum of squares	F-value	p-value
species	1	0.86	3.55	0.001
organ	1	1.56	6.45	1*10 <sup>-4</sup>
hour	1	1.82	7.57	1*10 <sup>-4</sup>
species*organ	1	0.67	2.78	0.007
species*hour	1	0.34	1.42	0.16
organ*hour	1	0.53	2.21	0.02
triple interaction	1	0.33	1.38	0.18
residuals	32	7.72		

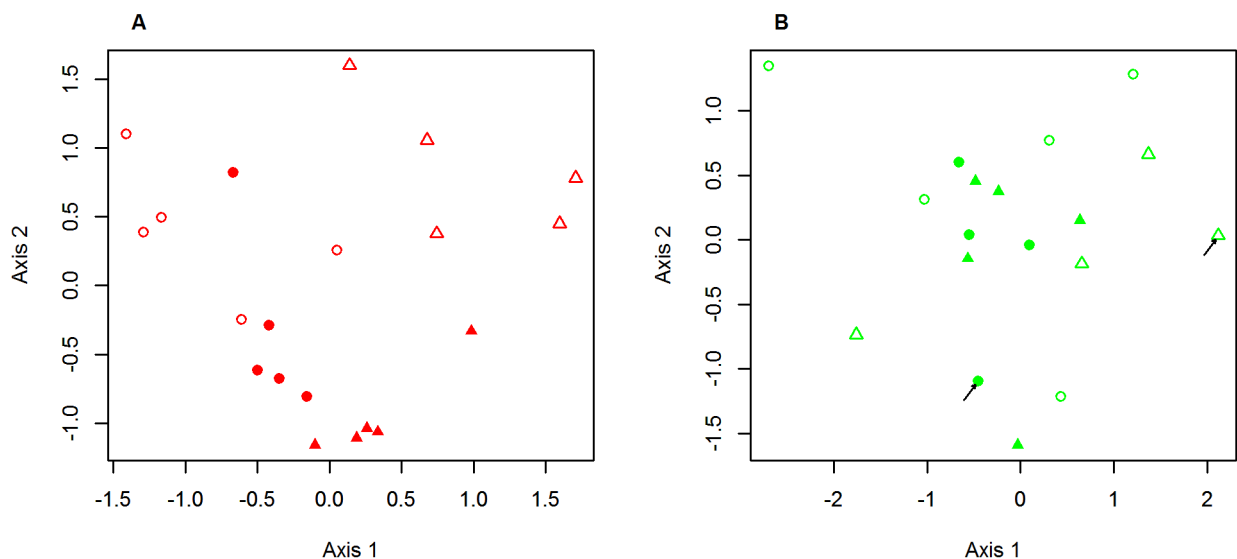
doi:10.1371/journal.pone.0103581.t002

generalized increase in total monoterpenoid proportion at noon compared to sunrise scents, in figs of both species as well as in leaves (Table S3). In leaves, the relative proportion of (E)-DMNT was also higher at noon. This difference is due to a much stronger increase in the absolute quantity of several monoterpenes (and of (E)-DMNT in leaves) between sunrise and noon samples relative to other VOC categories (not shown). Consistent with the orthogonality of species and hour of extraction effects on fig scent composition, a set of sesquiterpenes were responsible for a large part of the difference in scent composition between figs of both species (Table S4).

## Discussion

While it is rarely mentioned in the literature, field evidence shows that light colored fig pollinating wasps are generally nocturnal and dark colored fig pollinating wasps are diurnal [49]. To our knowledge, our results provide the first demonstration of the occurrence of a diurnal light colored fig pollinating wasp. This light colored fig pollinating wasp co-occurs on its host

tree with a very closely related species that is dark colored. Studies using molecular markers have allowed detecting numerous cases in which several fig wasp species, generally qualified as cryptic, pollinate the same host [6]. We provide here one more example in which it is demonstrated that co-pollinators present divergent ecological traits, a feature which should facilitate co-existence. We suggest that most cases of several species of fig pollinating wasps co-occurring locally on a host will turn out to correspond either to contact zones between different species or to the co-occurrence of species presenting strongly divergent ecological traits. We show that the black pollinator species associated with *Ficus septica* is longer lived than the yellow species a feature which should enable it to drift further in the wind in search of receptive figs, and hence reach receptive figs located further away from the one they were born in. This should give some advantage to the black species comparatively to the yellow one. The two species coexist throughout the island of Luzon, and a similar situation is found in Taiwan with a yellow and a black species associated with *F. septica* [18]. This widespread coexistence can only be explained if some trait of the yellow species compensates for its shorter survival.



**Figure 5. Variation in the composition of the scents emitted by figs (A) and leaves (B).** Two-dimensional NMSD ordinations on relative scent composition (% each VOC) computed separately on (A) fig scents (stress-value = 14%) and (B) leaf scents (stress-value = 11%). Circles represent *Ficus nota* and triangles *Ficus septica* samples, open symbols are sunrise samples and closed symbols are noon samples. Black arrows indicate places where two samples of the same category share the same locus.  
doi:10.1371/journal.pone.0103581.g005

**Table 3.** Refined PERMANOVA analysis on the relative composition of scents emitted by figs of *Ficus septica* and of *Ficus nota* at sunrise and at noon.

Factor	Df	sum of squares	F-value	p-value
species	1	0.97	4.79	0.0005
hour	1	1.16	5.72	0.0002
species*hour	1	0.39	1.93	0.072
residuals	16	3.26	0.56	

doi:10.1371/journal.pone.0103581.t003

We therefore predict that the yellow species is more efficient than the black species at rapidly locating, entering and/or ovipositing in receptive figs. If this competitiveness-lifespan trade-off hypothesis holds true, we would expect the black species to be more abundant in places where *Ficus septica* population density is low and the yellow species to be more abundant where population density is high. Hence, their coexistence would be facilitated by spatial heterogeneity of density of the resource they compete for. While *Ficus septica* fruits throughout the year, its fruiting frequency varies across seasons [50]. Therefore, we predict that the identity of the favoured species at a given location may also vary throughout the year.

We found published data and species descriptions suggesting the presence of the same pattern for the wasps associated with two other *Ficus* species: *F. rubiginosa* (subgenus *Urostigma* section *Malvanthera*) and wasp genus *Pleistodontes* in Australia, and *F. tuerkheimii* (subgenus *Urostigma* section *Americana*) and wasp genus *Pegoscopus* in America [14–17]. There are therefore potentially three independently evolved cases of diurnal light colored fig pollinating wasps pollinating a fig tree in competition with a very closely related black species. If diurnal behavior is confirmed, then it will be possible to test the prediction that in all three cases, the yellow species is shorter lived than the black one. Further investigations would then be needed to establish precisely what are the traits enabling yellow species persistence despite their shorter lifespan.

Other cases of *Ficus* species colonized by light colored and dark colored fig wasps are strikingly different. They include some species of section *Galoglychia* pollinated by genera *Alfonsiella* and *Elisabethiella* [7]. But in those cases the *Alfonsiella* species are light colored and nocturnal and the *Elisabethiella* species are dark and diurnal. Similarly, *Ficus sycomorus* is visited by two agaonid wasps of genus *Ceratosolen*. *C. arabicus*, the pollinator, is light colored and nocturnal while the distantly related *C. galili* does not carry pollen and is dark colored and diurnal [26].

In order to establish whether the two wasp species pollinating *Ficus septica* encountered the same receptive fig scents, we analyzed daily variation of receptive fig scent composition in *F.*

*septica* and in another fig species, *F. nota*, as a control. In both species the quantity of volatile compounds produced increased between morning and noon. Further the composition of the scent varied, notably the monoterpene content increased, i.e. compounds that are known to be detected by insects and among them fig wasps [38–39] and that are known to be used by insects to locate flowers. Hence, despite the central role of receptive fig scent in attracting fig wasps [28–31], this has not led to highly stereotyped receptive fig scents throughout the day. This new result is in agreement with previous studies which have evidenced strong variation within population among trees in receptive fig scent composition (one exception, *F. semicordata* uses a private channel to attract its pollinator [51]). Because of 1) the similar patterns of scent emission by the two *Ficus* species and 2) the similitude between figs and leaves in the daily pattern of scent variation, and 3) because monoterpenes are known to protect plant tissues against temperature and oxidative stresses [35,36], we suggest that this variation is, at least in part, due to the production of volatile compounds protecting the organs against temperature and oxidative stresses. The biology of *Ficus sycomorus* suggests that the tree has limited control on the production of volatiles used by wasps to detect receptive figs. Indeed, *F. sycomorus* is pollinated at night by *Ceratosolen arabicus*, and in the daytime it is colonized by *C. galili*, a species that does not provide any pollination service. Despite this pattern of visitation, *F. sycomorus* receptive figs produced at noon the same main volatile compound and similar total quantities of volatile compounds as the closely related diurnally pollinated *F. sur* [31].

In this study, we demonstrated that black and yellow pollinating wasps of *Ficus septica* have different lifespan and that receptive fig scent composition varies during the day. The two wasp species are therefore submitted to somewhat different ranges of receptive fig scents. We may therefore expect that they use a somewhat different range of chemical cues to locate receptive figs. If this is the case, then we may speculate on whether they respond differently to the within population variation of receptive fig scent among individual trees. Any such variation could lead to some assortative mating of the fig trees, thus structuring the gene flow

**Table 4.** Refined PERMANOVA analysis on the relative composition of scents emitted by leaves of *Ficus septica* and of *Ficus nota* at sunrise and at noon.

Factor	Df	sum of squares	F-value	p-value
species	1	0.47	1.72	0.10
hour	1	1.04	3.78	0.0015
species*hour	1	0.3	1.09	0.37
residuals	16	4.4	0.71	

doi:10.1371/journal.pone.0103581.t004

**Table 5.** Main VOC responsible for the difference between sunrise and noon scent composition in figs of each species and in leaves.

	<i>F. nota</i> figs	<i>F. septica</i> figs	Leaves
<i>monoterpenes</i>			
sabinene	*	ns	ns
myrcene	*	*	ns
$\alpha$ -terpinene	*	ns	ns
1,8-cineole	*	ns	ns
(Z)- $\beta$ -ocimene	*	**	*
(E)- $\beta$ -ocimene	*	*	*
$\gamma$ -terpinene	*	ns	ns
menthatriene	ns	*	ns
allo-ocimene	ns	**	*
<i>irregular terpenes</i>			
(Z)-DMNT	ns	ns	**
(E)-DMNT	ns	**	**
<i>phenylpropanoids/benzenoids</i>			
Aromatic 1	*	*	*
p-cymene	*	ns	ns
aromatic 2	*	ns	ns
p-cymenene	ns	*	ns
aromatic 5	ns	**	*
<i>unidentified</i>			
unidentified alcohol	ns	*	ns

P-values for the Mann-Whitney tests comparing the mean relative contribution of individual VOC to sunrise and to noon scent samples. Significance codes (values non corrected for multiple testing): ns = non-significant, \* < 0.05, \*\* < 0.01.

doi:10.1371/journal.pone.0103581.t005

within the *Ficus* species. Whether such processes are at work and whether they could ultimately lead to host speciation is an open question.

## Supporting Information

**Table S1 The daily rhythm of pollinator activity around trees bearing receptive figs: detailed results.** (DOCX)

**Table S2 Tree by tree results of the experiment where the accessibility of receptive *Ficus septica* figs was manipulated.** Number of yellow and black foundresses found inside receptive figs that have been accessible to pollination either for the whole day or in the afternoon only. (DOCX)

**Table S3 Mean relative composition of the scents emitted by figs and leaves of *Ficus septica* and *Ficus nota* at sunrise and at noon.** Mean relative contribution of each VOC to the scent of each sample category, expressed as a mean percentage  $\pm$  standard deviation. (DOCX)

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**Table S4 Main VOC responsible for additional differences of interest.** P-values for the Mann-Whitney tests comparing the mean relative contribution of individual VOC between categories indicated by column titles. Each comparison is between groups of 10 samples. Significance codes (values non corrected for multiple testing): ns = non significant, \* < 0.05, \*\* < 0.01, \*\*\* < 0.001. (DOCX)

## Acknowledgments

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## Author Contributions

Conceived and designed the experiments: L. Conchou LR FK. Performed the experiments: L. Conchou L. Cabioch LR. Analyzed the data: L. Conchou L. Cabioch. Contributed reagents/materials/analysis tools: L. Conchou LR FK. Contributed to the writing of the manuscript: L. Conchou L. Cabioch LR FK.

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# Appendix 4. Poster presented at the 6<sup>th</sup> European Phycological Congress



## Chemical signaling and defense in brown algal kelps during interactions with herbivores

*Cabioch Léa<sup>1,2</sup>, Ritter Andrés<sup>1</sup>, Goulitquer Sophie<sup>1</sup>, Potin Philippe<sup>1</sup>, Faugeron Sylvain<sup>2</sup>, Leblanc Catherine<sup>1</sup>*

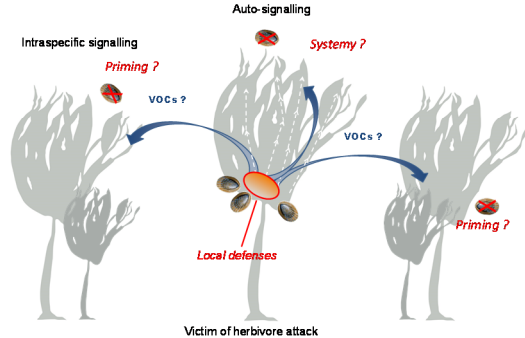
1- Sorbonne Université, UPMC Univ Paris 06, CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France  
2- Departamento de Ecología, Pontificia Universidad Católica de Chile, Santiago de Chile, Chile

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### Introduction

In the terrestrial environment, **volatile organic compounds**, such as aldehydes, are emitted by plants following herbivore's attack, and perceived by neighboring plants as **warning distance signals**, leading to priming and systemic defense. Marine algae actively respond to biotic stress, such as grazer's attack, by regulating transcription and metabolic pathways. We have recently shown systemic responses in the brown alga *Laminaria digitata* upon defense elicitation by oligoguluronates (1) and demonstrated a priming effect, induced by **chemical cues** present in seawater surrounding a kelp bed and/or released from neighboring brown algae (2). In the context of **kelp/herbivore interactions**, we are developing metabolomic approaches to identify the chemical signals involved in the defense upon herbivory in two emblematic kelp species, *L. digitata* from North-Atlantic Brittany and *Lessonia spicata* from Chile.



VOCs = Volatile organic compounds

Hypothetical defense signaling during kelp/herbivore interactions

---

### Methodology

**External signal**

- Grazers
- Co-incubated alga
- Oligoguluronates
- Aldehydes (4-HHE, 4-HNE, Dodecadienal)

**Impacts**


- Interactions with herbivores
- Algal distance signaling
- Algal defense metabolic pathways

**Monitoring**


- Effects on algal deterrence
- Exo-metabolome / potential priming inducers
- Endo-metabolome

**Methods**

- Bio-assays with specialist herbivores
- LC-MS analyses
- GC-MS analyses
- Galaxy (3)



*Laminaria digitata*  
*Patella pellucida*

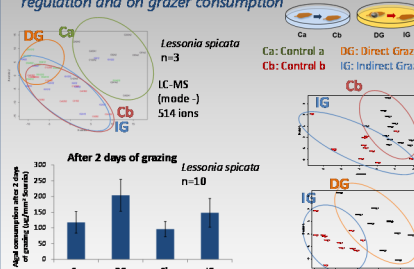


*Lessonia spicata*  
*Scurria scurra*

---

### Results

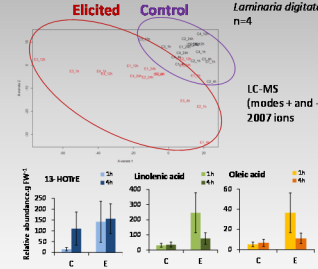
*Effects of direct and indirect grazing on algal metabolism regulation and on grazer consumption*



**After 2 days of grazing**  
*Lessonia spicata* n=10

- ✓ Metabolomic analyses show that grazing by herbivores (DG) induces modifications of the endo-metabolome, as well as co-incubation with a grazed alga (IG ≠ Cb, IG ≠ DG).
- ✓ Moreover, the bio-assay shows that previous grazing or co-incubation with a grazed alga seem to increase subsequent algal consumption by herbivores.

*Kinetics of the exo metabolome of an elicited alga*

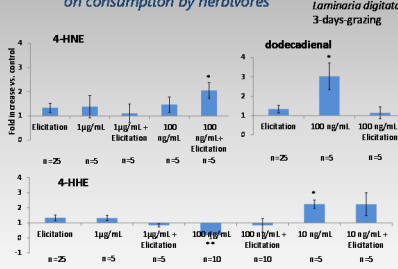


**Laminaria digitata** n=4

LC-MS (modes + and -) 2007 ions

- ✓ Statistical analysis (PLS-DA) shows that defense elicitation by oligoguluronates significantly modifies the exo-metabolome released by kelps in seawater.
- ✓ Upon defense elicitation, fatty acids and oxylipins are rapidly over-produced and released in seawater after 1h.

*Effects of algal incubation with an aldehyde on consumption by herbivores*



**Laminaria digitata** 3-days-grazing

- ✓ Chemical compounds, like the aldehydes 4-HNE or dodecadienal, tend to increase grazing impacts by herbivores.
- ✓ However, incubation of algae in the aldehyde 4-HHE at 100 ng/mL significantly decreases algal consumption by herbivores, and seems to confer a better protection against grazing.

---

### Conclusions and perspectives

Upon grazing by herbivores and perception of waterborne chemical cues emitted by a grazed alga, **specific metabolic pathways** seem to be induced in a neighboring kelp. This appears to increase subsequent grazing after a previous grazing or perception of chemical compounds. Moreover, defense elicitation induces the release of numerous **waterborne compounds** in seawater, such as **fatty acids and oxylipins**, or aldehydes. The aldehyde 4-HHE, at the concentration of 100 ng/mL, seems to trigger defense responses or **priming** state, leading to a **better protection against grazers**. The chemical identification of waterborne cues in seawater and differentially-expressed compounds in the algae should lead to a better understanding of the metabolic regulations occurring during defense and distance signaling in kelps.

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## **Appendix 5. Training**

- Expertise Flore, 10-31 juillet 2013, Roscoff
- Anglais « Scientific Writing », 23-24 janvier 2014, Paris (2 jours)
- Traitement de données métabolomiques sous Galaxy, 28 janvier 2014, Roscoff (1 jour)
- Linux, 12 mai 2014, Roscoff (1 jour)
- Cluster, 15 mai 2014, Roscoff (1 jour)
- Cycle "Projet Professionnel et Gestion de Carrière", 20-24 avril 2015, Roscoff (4 jours)
- Ecole Workflow4metabolomics, 21-25 septembre 2015 , Roscoff (5 jours)
- Stage "Vulgariser les sciences", 7-9 mars 2016, Orsay (3 jours)
- Journée Entreprise "les métiers de la culture", 13 juin 2016, Paris (1 jour)

## Appendix 6. Conferences

Journées Idealg	Roscoff	October 21 <sup>st</sup> -23 <sup>rd</sup> , 2013	Poster <b>2nd poster price</b>
AG GdR BioChimar	Lorient	November 7 <sup>th</sup> , 2013	
Journée de la plateforme Corsaire	Rennes	November 8 <sup>th</sup> , 2013	
Journée des Jeunes Chercheurs de la Station Biologique de Roscoff	Roscoff	December 5 <sup>th</sup> , 2013	Poster <b>1st poster price</b>
Journées de la Société Phycologique de France	Roscoff	December 16-18 <sup>th</sup> , 2013	
Ecole Thématique en Ecologie Chimique (ETEC)	Marseille	June 16-20 <sup>th</sup> , 2014	
Journées du Réseau Français de Metabolomique et de Fluxomique (RFMF)	Lille	June 9-11 <sup>th</sup> , 2015	Poster <b>1st student poster price</b>
6th European Phycological Congress	London	August 23-28 <sup>th</sup> , 2015	Poster <b>1st poster price</b>
Journées de la Société Phycologique de France	Vannes	September 23-25 <sup>th</sup> , 2015	Poster
2è journées scientifiques du GdR MediatEC	Banyuls-sur-Mer	October 28-30 <sup>th</sup> , 2015	Oral communication
Journées Idealg	Roscoff	November 5-6 <sup>th</sup> , 2015	Oral communication
Workshop CeBiB (Centro de Biotecnología y Bioingeniería)	Santa Cruz, Chile	December 2 <sup>nd</sup> -4 <sup>th</sup> , 2015	Poster
International Conference on Ecological Sciences – Sfécologie 2016	Marseille	October 24-27 <sup>th</sup> , 2016	Oral communication
3è journées scientifiques du GdR MediatEC	Marseille	October 28-29 <sup>th</sup> , 2016	

## Appendix 7. Other participations

- Membre élue du collège étudiant au Conseil d'Administration de la Station Biologique de Roscoff (2014 – 2016)
- Suppléante du collège étudiant au Conseil de Laboratoire de l'UMR 8227 (2014 – 2016)
- Présidente de l'Association Française des Jeunes Chercheurs en Ecologie Chimique (AFJCEC), oct 2014 – oct 2015.
- Organisation de la Journée des Jeunes Chercheurs de la Station Biologique de Roscoff, décembre 2014.
- Participation au Dance Your PhD contest (2015) "Chemical signaling in brown macroalgae during herbivory"
- Participation à l'organisation des 2èmes journées scientifiques du GdR MediatEC (2015).
- Participation à la Fête de la Science, Station Biologique de Roscoff (2014, 2015)
- Nuit des Chercheurs, Océanopolis, Brest (2014, 2015)
- Participation au stand Macroalgues sur le Quai des Sciences, Fêtes Maritimes de Brest 2016 (juillet 2016)





## Abstract

Kelps are founding species of temperate marine ecosystems, they evolve in the intertidal coastal areas where they are often challenged by herbivores. As most of sessile organisms, kelps develop defensive strategies against herbivory. In the kelp *Laminaria digitata*, elicitation by oligoguluronates induced regulations of transcription and metabolic pathways, leading to production and release of metabolites, such as aldehydes, in the environment. Moreover, this kelp species feature systemic responses upon defense elicitation and waterborne compounds surrounding a kelp bed have shown to modify its defense physiology. Such distant chemical signaling during defense responses are reminiscent of systemic and priming mechanisms in plants. In marine kelp beds, it should have a major ecological role in structuring marine algal and herbivore communities.

In the context of kelp/herbivore interactions, we investigated the defense responses and signaling processes in two emblematic kelp species from north Atlantic and South Pacific coasts, involved in specialized interactions with their herbivores, *L. digitata* in interaction with *Patella pellucida* (Brittany, France) and *Lessonia spicata* with *Scurria scurra* (Chile). Combined approaches of metabolomics and bio-assays were carried out to decipher the nature of the putative chemical cues, and their biological and ecological roles in algal defense against herbivores.

Results showed that, in response to grazing, kelps featured metabolic modifications such as induction of fatty acids, oxylipins and amino acids biosynthetic pathways, as well as transcriptomic modifications. Moreover, we observed that kelps co-incubated with grazed algae exhibited endo-metabolome changes compared to grazed one, and seemed to be less consumed during a subsequent grazing, suggesting the perception and integration of herbivory-induced algal compounds in neighboring algae. As field and lab studies previously highlighted a strong release of aldehydes into the air and the seawater following biotic and abiotic stresses, we further explored the biological roles of aldehyde-based signaling in kelp/herbivore interactions. We found that aldehydes could modify algal metabolism, including the activation of fatty acid metabolism, and that the application of 4-HHE on *L. digitata* could significantly reduce later algal consumption by herbivores. Thus, these results contribute to a better understanding of defense responses following grazing in kelps, and revealed the occurrence of distance signaling during interaction with herbivores, which could involve algal emitted compounds, such as aldehydes.

## Résumé

Les kelps sont des espèces clés dans les écosystèmes marins tempérés, elles vivent en milieu intertidal le long des côtes où elles sont souvent exposées à des herbivores. Comme la plupart des organismes sessiles, les kelps ont développé des stratégies de défense contre l'herbivorie. Chez la laminaire *Laminaria digitata*, l'élicitation par les oligoguluronates induit des régulations transcriptomiques et l'activation de voies métaboliques, menant à la production et la libération de métabolites dans l'environnement, tels que des aldéhydes. De plus, cette espèce d'algue brune a montré des réponses systémiques suite à une élicitation. Des composés solubles dans l'eau de mer environnant les forêts de laminaires peuvent aussi modifier leur physiologie en réponse à l'élicitation. Une telle signalisation chimique à distance lors des réponses de défense rappelle les phénomènes de protection systémique et de potentialisation chez les plantes. Dans les forêts de laminaires, celle-ci pourrait jouer un rôle écologique majeur dans la structuration des algues marines et des communautés d'herbivores.

Dans le contexte des interactions kelp/herbivores, nous avons étudié les réponses de défense et les processus de signalisation chez deux espèces emblématiques de laminaires originaires des côtes atlantiques et du Pacifique Sud, impliquées dans des interactions spécialisées avec leurs herbivores, *L. digitata* en interaction avec *Patella pellucida* (Bretagne, France) et *Lessonia spicata* avec *Scurria scurra* (Chili). Des approches combinées de métabolomique et de bio-essais ont été réalisées pour élucider la nature de potentiels signaux chimiques et leurs rôles biologiques et écologiques dans la défense des algues face aux herbivores.

Les résultats ont montré que, en réponse à un broutage, les kelps présentent des modifications métaboliques telles que l'induction de voies de biosynthèse des acides gras, des oxylipines et d'acides aminés, ainsi que des modifications transcriptomiques. De plus, nous avons constaté que les laminaires co-incubées avec des algues broutées présentaient des changements de leur endo-métabolome en comparaison à des algues broutées, et semblaient moins consommées lors d'un broutage ultérieur, suggérant une perception et une intégration des composés algaux induits par l'herbivorie par les algues voisines. Des études de terrain et en laboratoire ayant précédemment mis en évidence une libération massive d'aldéhydes dans l'air et l'eau de mer après des stress biotiques et abiotiques, nous avons exploré les rôles biologiques d'une signalisation de type aldéhyde dans les interactions kelp/herbivores. Nous avons montré que les aldéhydes pouvaient modifier le métabolisme d'une algue par l'activation du métabolisme des acides gras, et que l'application de 4-HHE sur *L. digitata* pouvait réduire significativement une consommation algale ultérieure par les herbivores. Ainsi, ces résultats participent à une meilleure compréhension des réponses de défense suite à un broutage chez les laminaires, et confortent l'existence d'une signalisation à distance dans les interactions avec les herbivores, qui impliquerait des composés de défense émis par les algues, dont les aldéhydes.