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**INDUCTION OF ANTI-HERBIVORE DEFENCES BY DIRECT  
AMPHIPOD CONSUMPTION AND WATER BORNE GRAZER  
CUES IN MACROALGAE**

(dissertação para a obtenção do grau de mestre em Estudos Marinhos e  
Costeiros)

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# Induction of anti-herbivore defences by direct amphipod consumption and water borne grazer cues in macroalgae

## ABSTRACT

Herbivores have been repeatedly shown to induce defences in terrestrial plants, but few examples exist from marine macroalgae. In two bio-assayed laboratory experiments we tested the effects of (1) direct amphipod consumption, (2) water borne cues from nearby grazed conspecifics, and (3) water-borne cues from non grazing consumers on the induction of anti-herbivore defences in macroalgae of the southern coast of Portugal. Both the brown alga *Fucus vesiculosus* and the red alga *Sphaerococcus coronopifolius* displayed distinct defence regulation. They induced defences when consumers were present and reduced defences again when consumption ceased. A reduction in palatability was considered to be indicative of an alga's defence mechanism. Defence regulation reacted to consumption stimuli in less than two weeks. For *F. vesiculosus* we found the defence activity in the lipophilic fraction of the alga's chemistry. Both algal species induce defences after receiving water-borne signals from nearby grazed conspecifics as well as non-grazing amphipods. These results imply that species of red and brown algae can recognise a suite of cues indicating herbivore pressure, allowing them to adjust their defensive traits in finely tuned short-term responses. Inducible defences may be more common in macroalgae than suspected.

**Key Words:** Herbivore induced defences, Marine algae, Water borne cues, *Fucus vesiculosus*, *Sphaerococcus coronopifolius*

# Indução de defesas em macroalgas contra a herbivoria através do consumo directo de anfípodes e de sinais propagados pela água

## RESUMO

A herbivoria é um factor chave para controlar a biomassa e a estrutura das comunidades de macroalgas. De modo a evitar a intensa herbivoria, as macroalgas são capazes de utilizar diversas estratégias como resposta ao ataque dos herbívoros. Sendo assim, as macroalgas podem evitar, tolerar ou defenderem-se dos consumidores. As defesas podem ser de quatro tipos: estruturais, por associação, nutricionais ou defesas químicas. Esta última consiste no uso de compostos de reduzida palatabilidade, tóxicos ou repulsivos para herbívoros, sendo a sua maioria metabolitos secundários. A defesa química das algas pode ser constitutiva, i.e. permanentemente disponível, ou induzida, i.e. produzida quando necessária. Ambas as estratégias de defesa têm as suas vantagens e desvantagens. Apesar das defesas constitutivas assegurarem uma protecção permanente (desde que os herbívoros não se adaptem à defesa imposta), estas podem corresponder a custos energéticos desnecessários para a alga quando os consumidores estão ausentes. No entanto, a importância dos custos das defesas é um tema ainda em discussão e provavelmente difere entre espécies, compostos de defesa e condições ambientais. As defesas induzidas poderão minimizar estes custos mantendo as defesas baixas até serem realmente necessárias. Contudo, a protecção poderá vir atrasada relativamente à percepção de um estímulo apropriado. Se o sinal for a danificação do tecido, a alga irá perder biomassa antes do consumidor ser repellido. Deste modo, os meso-herbívoros como os anfípodes, isópodes ou gastrópodes, com uma locomoção mais lenta e consumindo pequenas porções de alga numa escala temporal maior, podem favorecer o tipo de defesa induzida. Na presença de macro-herbívoros como os peixes, que atacam de uma maneira mais repentina, intensa e menos permanente, uma defesa constitutiva será a mais indicada. No caso de defesas induzidas, após a ameaça do herbívoro, estas devem consequentemente baixar. Alguns estudos realizados em macroalgas mostraram que as defesas cessaram aproximadamente após duas semanas a seguir ao contacto com os herbívoros. Ao contrário do que acontece para as plantas terrestres, são conhecidos poucos exemplos de defesa induzida contra a herbivoria nas macroalgas. Este facto poderá ser devido ao número reduzido de estudos realizados para estes organismos quando comparado com as plantas terrestres. Até agora, a indução de metabolitos secundários nas macroalgas como resposta ao consumo dos meso-herbívoros foi maioritariamente encontrada em algas castanhas (e.g. *Dictyota menstrualis*, *Sargassum filipendula*, *Fucus vesiculosus*), havendo já alguns exemplos para algas vermelhas (*Pterocladia capillacea*, *Hypnea pannosa*, *Galaxaura diessingiana*). Para além de defesas induzidas pelo consumo directo dos herbívoros, algumas plantas terrestres e organismos aquáticos são capazes de detectar sinais propagados pelo ar ou pela água. Só alguns estudos demonstraram os efeitos de sinais transmitidos através da água na indução de defesas anti-herbívoros em macroalgas (*Ascophyllum nodosum*, *Glossophora kunthii* e *Fucus vesiculosus*), no entanto, não está ainda esclarecido se os sinais provêm do herbívoro ou da alga.

O presente estudo teve como objectivos testar: (1) em que espécie de alga o consumo de anfípodes induz defesas, (2) se a existência de sinais propagados pela água provenientes de algas consumidas na vizinhança e/ou da presença de herbívoros não consumidores induzem defesas e (3) se a redução de defesas previamente induzidas ocorre após cessar a herbivoria.

A colheita das macroalgas realizou-se em dois locais, na costa rochosa de São Rafael, Albufeira (37° 05'N, 8° 15'W) e no sistema lagunar da Ria Formosa (37° 1'N, 7° 55'W) na costa sul Portuguesa. Nos estudos preliminares, foi testada a sobrevivência sob condições laboratoriais bem como a palatabilidade das algas. Desta forma, foram seleccionadas 7 espécies (*Cladostephus verticillatus* (Lightfoot), *Codium tomentosum* (Hudson), *Dictyopteris membranacea* (Stackhouse), *Fucus vesiculosus* (Linné), *Gelidium sesquipedale* (Clemente), *Ulva lactuca* (Linné) e *Sphaerococcus coronopifolius* (Stackhouse) para as experiências de indução. Todas as espécies de algas usadas são espécies comuns nas áreas de colheita. Para as experiências utilizou-se uma mistura de três anfípodas: *Gammarus insensibilis* (Stock), *Gammarella fucicola* (Leach) e *Cymadusa filosa* (Savigny), e na primeira experiência foi também utilizado o gastrópode *Bittium reticulatum* (Da Costa). As experiências decorreram no Laboratório Marítimo do Ramalhete, Universidade do Algarve, tendo sido a primeira iniciada em Julho e a segunda em Setembro de 2003, sendo ambas divididas em três fases de 2 semanas cada: aclimação, tratamento e recuperação. A primeira experiência teve como objectivo testar os efeitos da herbivoria na palatabilidade de cada espécie de alga, em dois níveis de tratamento: consumo directo (presença de herbívoros) e controlo (sem herbívoros). No início da experiência, as algas foram divididas em pequenos pedaços aproximadamente do mesmo tamanho. Alguns destes foram directamente congelados (a -20° C), e os restantes colocadas em aquários (com cerca de 2 l cada), para que durante a primeira fase (aclimação) as algas pudessem adaptar-se às condições de cultivo e recuperar de eventuais características defensivas adquiridas no seu ambiente natural. No final desta fase alguns pedaços de alga foram retirados dos aquários para uma posterior comparação das suas taxas de consumo (comparadas com algas inicialmente colocadas no congelador). Na fase de tratamento, em metade dos aquários foram adicionados herbívoros sendo a outra metade o controlo (n=5, para cada tratamento e espécie). No início da fase de recuperação, retiraram-se mais pedaços de algas e também os herbívoros de modo a testar se as defesas cessaram passadas 2 semanas de ausência de herbivoria. No final retiraram-se então as restantes algas para se proceder à comparação das taxas de consumo entre as algas que foram previamente sujeitas ao consumo directo de herbívoros, e as do controlo.

Na segunda experiência foram utilizadas apenas três espécies de algas (*Fucus vesiculosus*, *Sphaerococcus coronopifolius* e *Codium tomentosum*). O objectivo da experiência foi testar a existência de propagação na água de sinais provenientes de algas conspecificas a serem consumidas na vizinhança ou somente dos herbívoros, com capacidade de induzir defesas. Desta maneira foram utilizados quatro tratamentos: controlo, consumo directo, algas consumidas na proximidade e herbívoros não consumidores na proximidade. Para tal foi colocada uma rede (1 mm de malha) a dividir os aquários, sendo o resto do procedimento experimental totalmente idêntico ao da primeira experiência. No final de cada fase das duas experiências realizaram-se testes de preferência de alimentação, que consistiram em colocar os pedaços das algas de cada tratamento juntos numa caixa de Petri, sob o consumo directo de herbívoros, de modo a detectar diferenças entre o controlo e os tratamentos. Estes testes foram efectuados com algas intactas e também com alimento artificial. O alimento artificial consistiu num preparado constituído por diclorometano, um solvente orgânico responsável pela extracção dos compostos lipofílicos das algas testadas, e ainda água destilada e pó de *Ulva*. Este procedimento teve por objectivo verificar se as defesas induzidas provêm de metabolitos secundários não polares.

Na primeira experiência, após a fase de aclimação, não foram encontradas diferenças significativas nas taxas de consumo de algas intactas e alimento artificial, quando

comparadas as algas vindas directamente da natureza e as já aclimatizadas. Este resultado pode dever-se ao facto de quando as algas estavam no seu ambiente natural, poderem ter estado expostas a uma intensidade de herbivoria baixa ou mesmo ausente. Por outro lado, estas algas poderão ter um tipo de defesa constitutiva, apresentando um elevado nível de defesas. Após a fase de tratamento as algas controlo intactas foram, em geral, mais consumidas que os seus conspécíficos previamente consumidos. A alga castanha *Fucus vesiculosus* foi a única espécie que mostrou uma indução significativa de defesas. Em média, os pedaços de algas controlo intactas de *Fucus* foram 1.6 vezes mais consumidos que as algas previamente consumidas (teste-t emparelhado,  $t_4 = 4.83$ ,  $p < 0.05$ ). O alimento artificial produzido das algas controlo de *Fucus* foi em média 1.3 vezes mais consumido que as algas anteriormente consumidas (teste-t emparelhado,  $t_4 = 4.67$ ,  $p < 0.05$ ). Mais uma vez, o alimento artificial constituído pelas outras espécies estudadas não revelou diferenças significativas na palatabilidade no final da fase de tratamento. Estes resultados demonstram que a espécie *F. vesiculosus* poderá ter a capacidade de induzir metabolitos repulsivos ou tóxicos para os herbívoros, não pondo de parte a possibilidade de haver também uma indução de defesas morfológicas. No entanto, não foi observada nenhuma alteração morfológica aparente, como em alguns estudos de outros autores. Apesar dos resultados sugerirem que os compostos defensivos pertencem à fracção lipofílica dos metabolitos secundários, não se pode excluir a possibilidade de uma actividade defensiva adicional proveniente dos metabolitos polares, facto descrito em algumas experiências anteriores. Relativamente às outras espécies testadas, uma vez que não apresentaram qualquer efeito do consumo directo de herbívoros na sua palatabilidade, poderão dispor de uma defesa constitutiva em vez de uma defesa induzida. Outra hipótese para explicar os resultados poderá ser a existência de defesas específicas para diferentes espécies de herbívoros, ou até mesmo a necessidade de uma maior concentração destes. Após a fase de recuperação, já não foi detectado qualquer efeito de indução nas algas intactas de *Fucus*. As outras espécies de algas também não mostraram diferenças significativas nas taxas de consumo no final desta fase. Após as duas semanas na ausência de herbívoros, no final da fase de recuperação, a defesa induzida no *Fucus* baixou novamente e a palatabilidade do alimento artificial de indivíduos previamente consumidos já não difere do alimento constituído por algas controlo. Para as outras espécies também não foram encontradas diferenças significativas. Aparentemente a defesa da alga *F. vesiculosus* além de poder ser induzida, também apresenta capacidades redutoras no caso de cessar o consumo da parte dos herbívoros. Contudo, não é possível saber ao certo o dia em que estas defesas terminaram, apenas se sabe que houve um relaxamento destas durante os 14 dias. Deste modo, serão necessárias mais experiências para precisar o período no qual as defesas ainda se mantêm. Relativamente à segunda experiência, após a fase de aclimação nenhuma das três algas testadas (*S. coronopifolius*, *F. vesiculosus* e *C. tomentosum*) apresentou diferenças significativas nos níveis de palatabilidade, quer em algas intactas quer no alimento artificial. Em relação à alga verde *Codium tomentosum*, não houve qualquer diferença significativa nas taxas de consumo de algas intactas e alimento artificial no final das últimas duas fases da experiência. Este facto apoia os resultados obtidos na primeira experiência, indiciando que esta alga não tem defesas ou então tem defesas constitutivas. Os resultados relativos à espécie *Fucus vesiculosus*, mostraram que apenas as taxas de consumo entre algas controlo intactas e as expostas a sinais de algas a serem consumidas na proximidade e sinais unicamente de herbívoros foram significativamente diferentes. Só no final da fase de recuperação foram encontradas diferenças no tratamento com algas previamente expostas aos herbívoros, continuando a haver também preferência para algas controlo intactas relativamente às expostas a sinais

de herbívoros. Para o alimento artificial de *Fucus*, não foram detectados quaisquer efeitos na palatabilidade para os diferentes tipos de exposição à herbivoria, no final de ambas as últimas fases da experiência. Os padrões de indução foram alterados de duas formas em relação à primeira experiência. O atraso na resposta induzida pode ser resultado da sazonalidade variável da actividade da herbivoria, enquanto que os efeitos não detectados no alimento artificial poderão ser devidos a alterações no tipo de compostos químicos ao longo do ano. Após duas semanas de exposição aos herbívoros, os níveis de palatabilidade de algas intactas de *S. coronopifolius* expostas aos diferentes tipos de tratamentos foram significativamente reduzidos quando comparados com as algas controlo. Testes posteriores revelaram que as algas controlo desta espécie foram significativamente preferidas às (1) previamente expostas aos herbívoros, (2) às que receberam sinais de algas a serem consumidas na proximidade e (3) às que receberam sinais de herbívoros apenas. Na primeira experiência verificou-se uma tendência semelhante, no entanto os efeitos não foram significativos. Isto pode significar que a reacção ao consumo directo dos herbívoros pode depender da época do ano. Até à presente data, tem sido considerado que sinais da presença de herbívoros na vizinhança não são por si só suficientes para induzir uma resposta defensiva. Contudo, o presente estudo revela que as espécies *F. vesiculosus* e *S. coronopifolius* são sensíveis até mesmo na presença de anfípodes que não se encontram a consumir. Este é o primeiro exemplo de uma alga vermelha com a capacidade de induzir defesas através de sinais propagados pela água. Para algas estruturalmente menos complexas como as deste grupo, esta capacidade pode compensar a fraca transmissão de sinais entre as diferentes partes da mesma alga. No entanto, outros estudos já tinham demonstrado mecanismos de defesa induzida pelo contacto directo com herbívoros para outras espécies de algas vermelhas. No final da fase de recuperação, não houve diferenças significativas de taxas de consumo para os diferentes tratamentos. As taxas de consumo de alimento artificial desta espécie não foram significativamente afectadas pelos diferentes tipos de tratamento, quer no final da fase de tratamento quer após a fase de recuperação. Assim, as defesas induzidas poderão ser morfológicas ou químicas (fracção hidrofílica) excluindo os compostos não polares. Em conclusão, a eficiência dos compostos lipofílicos bem como o tempo de reacção e intensidade das defesas induzidas anti-herbivoria variaram ao longo do tempo.

**Palavras chave:** Defesa induzida pela herbivoria, Algas marinhas, Sinais propagados pela água, *Fucus vesiculosus*, *Sphaerococcus coronopifolius*

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# 1. Introduction

### *1.1. Macroalgae-herbivore interactions*

Marine benthic macroalgae play a key role in ecosystems (Costanza et al. 1997). Seaweeds provide up to 40% of the primary production in coastal zones, represent carbon-sinks (Smith 1981), and also provide dispersal stages with suitable settlement substrata (Rangeley and Kramer 1995, Gotceitas et al. 1997). In the decades, however, populations of perennial macroalgae tend to decrease in many coastal regions (e.g. Vogt and Schramm 1991, Meyer and Paul 1992, Schramm and Nienhuis 1996). A common explanation is the competitive advantage ephemerals and phytoplankton experience under eutrophic conditions (e.g. Duarte 1995). Depending on the food web structure of a system, mesoherbivores (Brawley 1992) may benefit from the presently observed overexploitation of predatory fish (Myers and Worm 2003), which would enhance their impact on macroalgae. Another reason could be due to weakened anti-herbivore defences of environmentally stressed macroalgae (e.g. Cronin and Hay 1996b). Herbivory can cause declines in macroalgal populations and represents a key factor for controlling biomass accrual and community structure of macroalgae (Lubchenco, 1978, Hay and Fenical 1988, Hay 1991, Cyr and Pace 1993), affecting then a central ecosystem service. Despite meso-herbivores low mobility and consumption rates relative to macro-herbivores (e.g. fishes), meso-herbivores (e.g. amphipods) due to their elevated numbers and more site-bound life histories may shape species composition of macroalgal communities (Brawley and Adey 1981, Duffy and Hay 2000). For example, herbivorous fish are reported to completely consume algal production in coral reefs (Hay 1997). In order to avoid the intense herbivory macroalgae are able to employ a variety of strategies to respond to herbivore attack. In that way to survive, they have the

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choice to escape from herbivores, to tolerate damage or defend themselves (Hay and Fenical 1988, Duffy and Hay 1990). Avoidance of consumption involves being where and/or when grazers are rare or inactive. Seaweeds can avoid herbivores spatially settling and growing in habitats that grazers fail to reach, e.g. by hiding in cracks and fissures, or temporally avoiding co-occurrence with important grazers by growing when predation pressures are low (Hay 1984, Duffy and Hay 1990). Tolerance to herbivory may be as compensatory growth (Lewis 1986, Hay 1997) or as spores that are able to survive gut passage (Buschmann and Santelices 1987), while mechanisms or strategies that increase the fitness of an alga when herbivores are present are usually called defence (Tollrian and Harvell 1999). Defending tissue against consumption involves making it less attractive to or shielding it from potential consumers. Defence is a common strategy among sessile organisms, especially ones that must grow in an exposed manner to acquire resources. Cronin (2001) reported four categories of defences used by seaweeds, (1) structural, (2) associational, (3) nutritional and (4) chemical:

(1) Structural defences involves the formation of protective arrangements, e.g. calcium carbonate ( $\text{CaCO}_3$ ) makes up as much as 90% of the dry mass of the hard thallus of seaweeds (calcareous red algae *Corallina officinalis* hardens its thallus by storing calcium carbonate in its tissue). It is not quite certain if the  $\text{CaCO}_3$  serves as structural defence by making the thallus harder to bite for the grazer or if the storage of  $\text{CaCO}_3$  might have an additional deterrent effect by diluting the nutritional value of the seaweed.

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(2) Associational defences occur when a species gains protection (mutualistic or not) from a natural enemy by associating with a protective host, e.g. bryozoans growing epiphytic on kelps is believed to be an example where the basibiont benefits from protection by the epibiont. Interestingly, several cases are known where seaweeds offer protection to mesograzers against larger predators (Hay et al. 1990, Brawley 1992, Hay 1997), but to the authors knowledge no example is known, where an alga benefits from protection by another alga.

(3) Nutritional defences act when the organism has a low nutritive value and consequently is protected from consumption because it is not worth eating, as it probably happens with *Corallina officinalis* explained above in (1).

(4) Chemical defences imply the use of compounds that are distasteful, toxic, or otherwise repulsive to consumers. Most defensive compounds are secondary metabolites (Hay and Fenical 1988). Seaweeds are similar to terrestrial plants in that they produce a variety of secondary metabolites, including terpenes, acetogenins, aromatic compounds, amino acid-derived substances, and phloroglucinol-based polyphenolics. They differ from terrestrial plants by incorporating halogens into some compounds and by rarely producing the nitrogen-containing alkaloids common in many terrestrial plants (Faulkner 1984, Hay and Fenical 1988). The diversity of algal chemistry is impressive. To date more than 5000 secondary metabolites have been described (in algae alone) of repellent secondary metabolites and spiny morphologies suggest.

### *1.2. Inducible versus constitutive defences*

Algal chemical defence can be constitutive, i.e. permanently available, or inducible, i.e. produced “when needed”. Both strategies do have their pros and cons. While constitutive defences do ensure a permanent protection (as long as the consumers do not adapt to the defence) they may incur unnecessary costs when consumers are absent. The importance of defence costs, however, is still under debate (Kearsley and Whitham 1992, Cronin 2001, Arnold and Targett 2003) and probably differs among species, compounds and environmental conditions. Characteristic constitutive traits are, for instance, structural barriers or the constant production of secondary metabolites (Agrawal and Karban 1999). Inducible defence, on the other hand, was defined as a phenotypic change that is triggered by cues associated with biotic agents (Tollrian and Harvell 1999). This type of defence could minimize putative costs by keeping the level of defence low until it is needed (Harvell 1990). On the other hand, in the case of defence induction protection will always lag behind the perception of an appropriate stimulus. If the cue is tissue damage, the algae will lose biomass before the consumer is deterred. If, however, the alga is capable of detecting the presence and/or feeding of the consumer in its vicinity this risk can be significantly reduced especially when the consumers are slow moving mesograzers or urchins.

After the threat of herbivory disappeared, defences should consequentially be reduced again. Isolated studies reported the down regulation of defences after a time span of nine to fourteen days, but they focused rather on induction than recovery. Renaud et al. (1990) stated that *Padina gymnospora* became less susceptible to grazing by the sea

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urchin *Arbacia punctulata* and subsequently lost this resistance after 9 days. Moreover, Peckol et al. (1996) found that increased levels of phlorotannins induced by simulated grazing in *Fucus vesiculosus* were reduced after two weeks. However, a study that standardises the examination of switch-off mechanism in bio-assayed induction experiments has, to the author's knowledge, not yet been conducted.

In order to predict in which conditions plants would benefit more from an induced rather than a constitutive defence, many factors have been discussed. Therefore, three basic requirements have to be met (Harvell 1990, Cronin 2001):

- Encounters with consumers must be unpredictable and infrequent.
- A reliable cue that indicates the presence of herbivores and predicts the probability of future consumption must be present.
- The defence must be effective in deterring herbivores.

The model shown in the Fig.1 demonstrates the reason of an inducible instead of constitutive defence.

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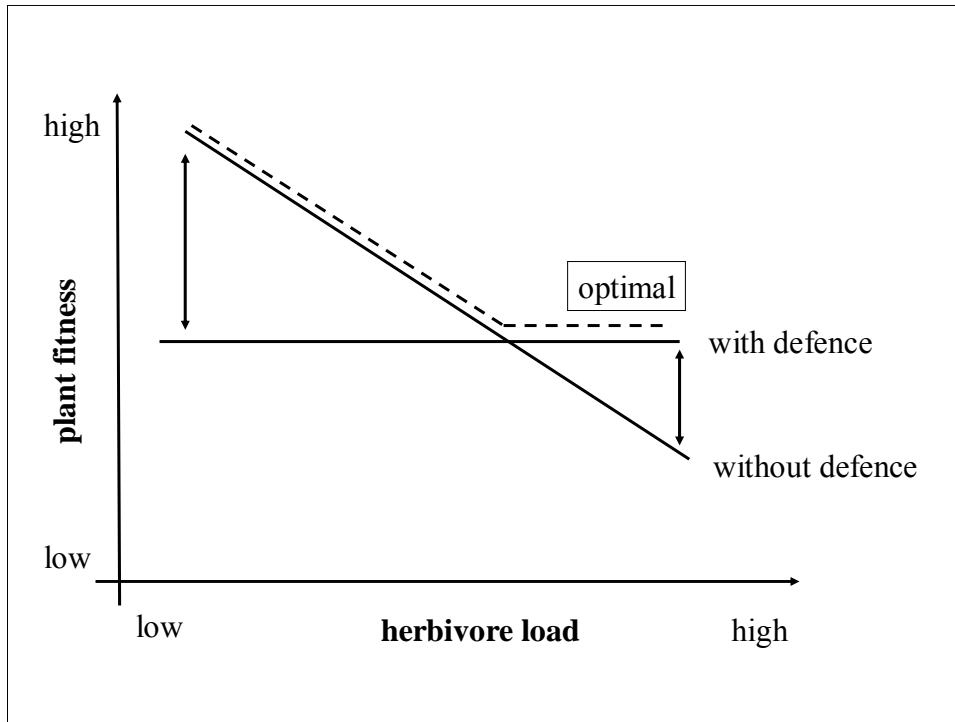


Figure 3 - The reason for induced defence.

The present model predicts that when herbivore densities are low, undefended plants will be in advantage compared to constitutively defended plants in terms of plant fitness. When the herbivore load increases, undefended individual will have a decrease of fitness, being committed without protection against grazing. Constitutively defended plants have an advantage in this case. However, the strategy of inducible defences combines both benefits and therefore should be the optimal strategy. To cope with a typically more sudden, more intense but less permanent attack by macro-herbivores such as fishes, a constitutive defence may be optimal. In contrast, meso-herbivores consume smaller portions of their food over longer time spans, which could favour the selection for defences that are adjusted to grazer pressure, like inducible defences (Hay 1996, Pavia and Toth 2000).

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With the aim of explaining the evolution of inducible defences, or by another words why it might be advantageous for a plant to defend itself only when the threat of herbivory is apparent, ecologists have discussed five main factors (Harvell 1990, Agrawal and Karban 1999):

- Defensive chemicals might be toxic for the plant itself. Therefore, the production of defences, only when they are required, would reduce the chance of auto-toxicity.
- Specialist herbivores might be able to detect certain waterborne cues. Avoidance of cue emission would make the algae more difficult to detect and therefore less susceptible.
- An induced response allows the plant to produce a qualitatively and quantitatively different response depending on the attacker.
- Fluctuating defence levels might be more difficult to adapt to and consequently slow down co-evolution of grazers.
- Defences on demand might save the plant costs in terms of photosynthate.

The production of chemical defences is believed to be costly (Hay and Fenical 1988, Cronin 2001) because such defences use resources that could have been allocated to growth or reproduction (Hay 1997, Duffy and Hay 2001). The notion of costs occupies

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a central position in most theories concerning inducible defences, since in addition to the principal energy spent producing defensive traits (that might not be necessary) consequential costs such as storage and maintenance can be avoided (Agrawal and Karban 1999). According to Harvell (1990), cost is usually estimated by measuring fitness components such as growth rate, reproductive output or survivorship. However, the interactions of these measures are extremely complex and difficult to assess. This is probably the reason, why the idea of defence being a trade-off for the individual has rarely been tested, even though is a very persuasive theory.

### *1.3. Induction experiments*

In contrast to terrestrial plants (Karban and Myers 1989, Karban and Baldwin 1997), only few examples of inducible antiherbivore defence exist for seaweeds (Van Alstyne 1988, Cronin and Hay 1996a, Pavia and Toth 2000, Toth and Pavia 2000). This is surprising, if we consider the approximate number of 80000 species of marine macroalgae worldwide reported to produce a rich variety of secondary metabolites (Pavia and Toth 2000), many of them known as anti-herbivore defences. This discrepancy may be due to the reduced research effort on seaweeds relative to terrestrial plants. Alternatively, terrestrial plants might possess better developed internal transport systems than, for instance, red and green seaweeds, which is suggested to be a necessary prerequisite to transmit localized induction cues, e.g. from single grazer bites, across whole plants (Cronin and Hay 1996a).

Previous attempts to induce defence in marine algae used artificial clipping to standardise consumption (Hammerstrom et al. 1998, Lowell et al. 1991, Pavia et al. 1997), but many of these studies failed to adequately mimic the natural grazing that would induce a response, in both terrestrial and marine plant-herbivore interactions (e.g. Baldwin 1990, Pavia and Toth 2000). Studies that have been successful in producing this type of defence (Van Alstyne 1988, Cronin and Hay 1996a, Pavia and Toth 2000) used a more ecologically relevant approach by using grazers.

Present knowledge suggests that anti-herbivory defences in algae are a quite differential response. For instance, Pavia and Toth (2000) demonstrated that grazer identity matters as to whether defences were induced or not. Moreover, algal responses may be tissue-

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specific, when valuable algal parts, e.g. holdfasts, display constitutive and other tissues inducible defences (Taylor et al. 2002). Seasonal patterns of induction add further complexity to alga-grazer interactions (Molis et al. 2006). Induced defences were shown to be temporally and spatially variable. For example, the defence levels in *F. vesiculosus* were lowered to pre-grazed levels within 10 days (Hemmi et al. 2004). In addition, the repulsive response was not spreading to adjacent tissue (Hemmi et al. 2004).

Until now, seaweed induction of secondary metabolites in response to mesograzer consumption was mainly found for brown algae, e.g.: *Dictyota menstrualis* (Cronin and Hay 1996a), *Sargassum filipendula* (Sotka et al. 2002), *Fucus vesiculosus* (Rhode et al. 2004). However, recent studies have found some potential red algae species that induced defensive responses against herbivores, e.g.: *Pterocladia capillacea* (Weidner et al. 2004), *Hypnea pannosa* (Ceh et al. 2005), *Galaxaura diessingiana* (Díaz et al. 2006).

#### ***1.4. Water-borne cues***

Besides inducible defence effects of direct grazing impacts, a number of terrestrial plants and aquatic organisms are able to sense air- and water borne cues, respectively (Dicke and Bruin 2001). It has been suggested that exudates released by neighbouring grazed plants, or indeed by the herbivores themselves, could induce a defensive synthesis in macroalgae (Toth and Pavia 2000, Sotka et al. 2002). Because seaweeds often form dense monospecific stands, where plants are in direct contact with each other and water flow is reduced, chemical signals can be easily transmitted between conspecifics. Individuals able to detect and respond to such cues might have a selection advantage, because they can defend themselves before an attack. The role of waterborne cues in the induction of defences has not yet been adequately studied. To date, only few studies have demonstrated the effects of water-borne cues in the context of inducing anti-herbivory defences in macroalgae (Toth and Pavia 2000, Rohde et al. 2004, Macaya et al. 2005). Toth and Pavia (2000) found an inducing activity of water borne cues in the brown alga *Ascophyllum nodosum* while Macaya et al. (2005) found in *Glossophora kunthii* and Rhode et al. (2004) in *Fucus vesiculosus*. However, knowledge on whether effective water-borne cues stem from the grazer or the alga is extremely scarce. As proposed by Toth and Pavia (2000), external chemical cues may be common elicitors for induced defence in nonvascular plants, i.e. red and green algae. On the other hand, Sotka et al. (2002) rejected this mechanism for the brown seaweed *Sargassum filipendula*.

*1.5. Aims of the present study*

In this study it was investigated whether grazing by amphipods could induce defences in two red, two green, and three brown macroalgae of the southern coast of Portugal. The aim of this study was to test:

- (1) in which algae grazing of amphipods induced defences,
- (2) whether the existence of water-borne cues from neighbouring grazed plants and/or the mere presence of a non-grazing consumer induces defences and
- (3) whether a reduction of previously induced defences occurred after herbivory ceased.

## **2. Collection sites**

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Collection sites were located on the rocky shore of São Rafael, Albufeira (37° 05' N, 8° 15' W, summer sea surface temperature reaching ca. 21 C, and salinity of 33 psu) and in the coastal lagoon-system of the Ria Formosa (37° 1' N, 7° 55' W, summer sea surface temperatures of 19-28 C, and salinity of 28-35 psu) on the Portuguese southern coast (Fig. 4).

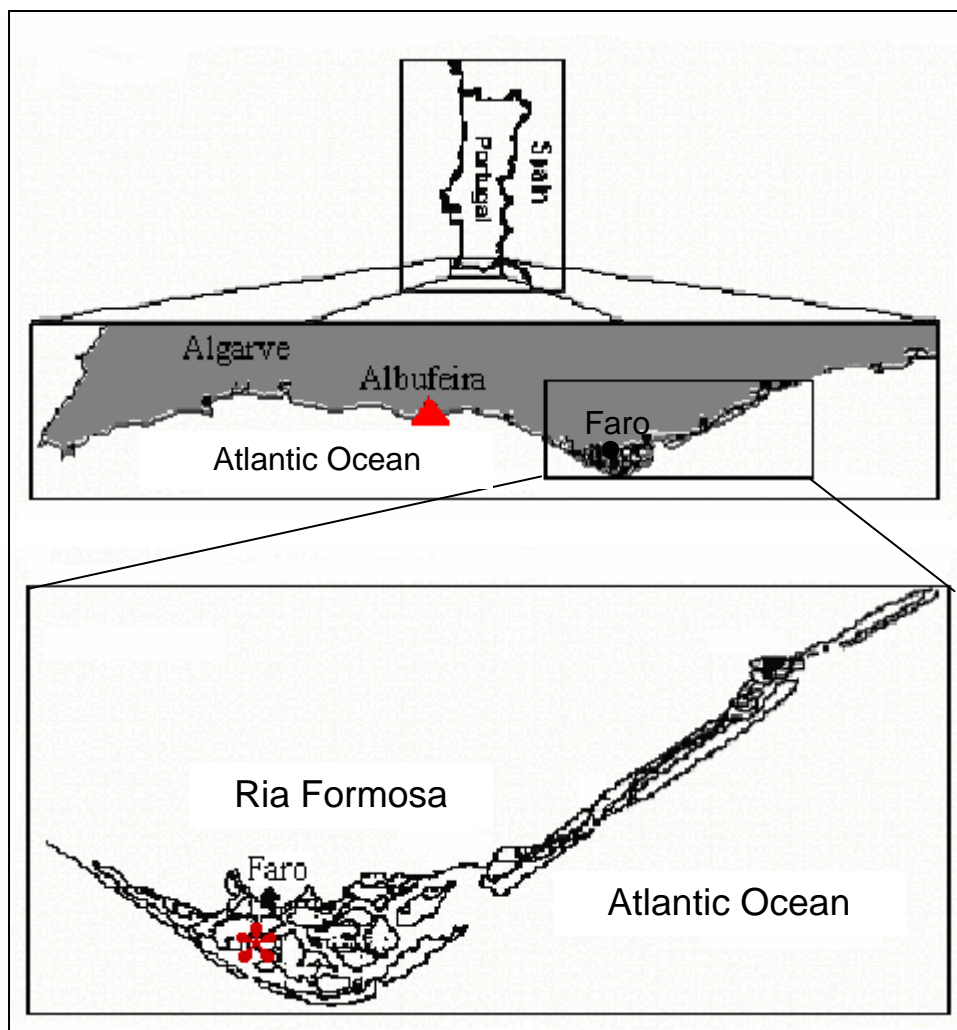


Figure 4 – Localization of the study sites ▲ – Praia de S.Rafael, Albufeira; \* - Ria Formosa, Faro.

The Ria Formosa lagoon system covers an area of 16.300 ha between a series of barrier island and the mainland along the southern tip of Portugal (Pilkey et al. 1989). As a result of tidal currents (average tidal range 1.5 and 3.7 m) the water body in the lagoon

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is well mixed. The water temperature in the summer ranges between 19 to 28°C and salinities from 35 to 28. The nutrient availability is high in winter but low in summer, in summer (June-August) there is a concentration of 0.04µM for nitrate and 1.25µM for phosphate (Newton 1995). A variety of different habitats can be identified based on substrate type (ranging from sand, gravel, to fine mud), depth, vegetation, and distance from the sea.

In the other collection area of Sao Rafael the sea surface temperature reaching values of 21°C or higher during the summer period. The wave conditions are less severe (>3m), since it is not exposed to the wave components predominant on the west coast. The nutrient supply on the south coast of Portugal is high, it reach values from 0.68 µmol/dm<sup>3</sup> for nitrate and 0.5 µmol/dm<sup>3</sup> for phosphate (Baptista 1997). Tidal range of spring tides are extreme is approximately of 3.5 to 4m along the Portuguese coast. The south west shore is dominated by bedrocks (Boaventura 2000).

# 3. Organisms

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In pilot studies, it was assessed survivorship of algae under cultivation conditions and performed feeding preference assays to determine which algae are readily consumed by grazers. Based on the consumption rates (For calculations see section 4.3. *Feeding preference assays with live algae*), it was selected seven species (*Cladostephus verticillatus* (Lightfoot), *Codium tomentosum* (Hudson), *Dictyopteris membranacea* (Stackhouse), *Fucus vesiculosus* (Linné), *Gelidium sesquipedale* (Clemente), *Ulva lactuca* (Linné) and *Sphaerococcus coronopifolius* (Stackhouse)) for the induction experiments. All algal species used in the experiment represented common species where they were collected (Table1).

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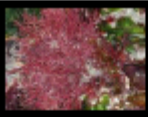



Algae class	Algae species	Substrat	Site	Stock density	Position on shore
Chlorophyceae	<i>Codium tomentosum</i> 	hs	RF	X	intertidal
	<i>Ulva lactuca</i> 	hs	RF	XX	intertidal
Rhodophyceae	<i>Gelidium sesquipedale</i> 	hs	SR	X	intertidal
	<i>Sphaerococcus coronopifolius</i> 	hs	SR	X	intertidal
Phaeophyceae	<i>Cladostephus verticillatus</i> 	hs	SR	XX	intertidal
	<i>Dictyopteris membranacea</i> 	hs	SR	XX	intertidal
	<i>Fucus vesiculosus</i> 	hs	RF	X	intertidal

Table 1: The 7 algae species for the induction experiment with a description of the collection areas. hs – hard substratum, RF – Ria Formosa, SR – Sao Rafael, X – high density, apart from each other 1 to 2 m, XX – high density

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Grazers used for the experiment were chosen because they were common in Ria Formosa, where they grazed on the studied algae and were found in high abundance. For the experiments it was used a mixture of three amphipods *Gammarus insensibilis* (Stock), *Gammarella fucicola* (Leach) and *Cymadusa filosa* (Savigny). In the first experiment the common snail *Bittium reticulatum* (Da Costa) was used, which was no longer found in high densities during the late summer months to be used in the second experiment, probably due to an increase of the water temperature.

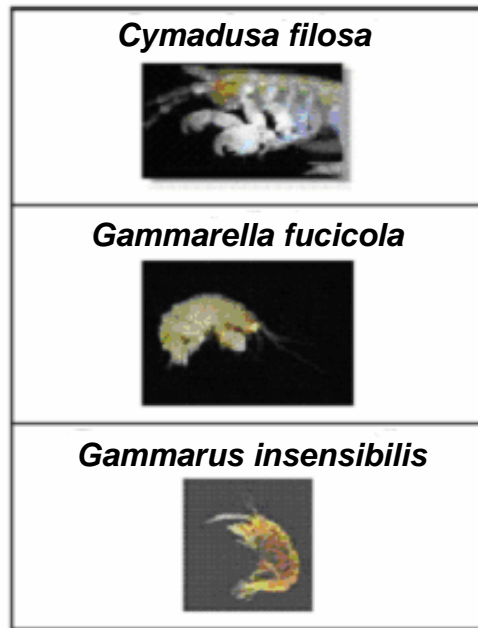


Figure 5 - The three amphipods used as consumers during the experiments.

## **4. Materials and methods**

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### *4.1. Pilot studies*

The pilot studies were conducted, as it was referred before, to determine which algae and herbivores were the most indicated to be used in these experiments. Thus, it was assessed the survivorship of the algae under cultivation conditions and performed feeding preference assays to determine which algae are readily consumed by grazers. For these preliminary experiments, nine species of algae (*Cladostephus verticillatus*, *Codium tomentosum*, *Sargassum vulgare*, *Dictyopteris membranacea*, *Fucus vesiculosus*, *Padina pavonia*, *Gelidium sesquipedale*, *Ulva lactuca* and *Sphaerococcus coronopifolius*) and five common consumers (*Bittium reticulatum*, *Gammarella fucicola*, *Gammarus insensibilis*, *Cymadusa filosa*, and *Gibbula* spp.) were tested. The herbivores were not starved before the experiments, since this could have effects on its feeding behaviour (Cronin and Hay 1996b). The algae were collected at the 17 June 2003 and transported in coolers to the laboratory in order to start the tests on the same day. For each combination tested (alga species + herbivore species) it was used a 3-fold replication. The algae were cut into three pieces (approx. 1.5 g each piece, depending on the alga species) to be used in the following three treatments:

- Direct consumption – with the presence of consumers
- Control – without consumers
- Growth control – without consumers

Each treatment took place in a 250 ml large plastic dish. For the direct consumption treatment it was used four to eight herbivores of the same species (depending on the

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alga species) in every replicate. The wet weight of each piece of alga was determined in the beginning and in the end of the assay in order to get the consumption rate. With the purpose of removing all additional water each piece was dried before weighing. The experiment last for ten days and every two to three days the water in the plastic dishes was replaced. Afterwards, the piece of the direct consumption treatment and the control piece were put together in the same plastic dish with four consumers for two days. In the end the wet weight was determined once more (see 4.4. *Feeding preference assays with live algae* to calculations). Out of these data the suitable algae and consumers were determined to be used in the induction experiments (Table 1).

***4.2. Induction Experiment Series - First experiment (Screening)***

A total of seven species of algae were tested for herbivore-induced defences in laboratory experiments at the Marine Laboratory of Ramalhete, University of Algarve, Faro, between July 2003 and October 2003. The first experiment (screening) tested the effects of the grazing on the palatability of each algal species. Two treatment levels were used: direct consumption (with the presence of herbivores) and control (without herbivores). Both treatments levels were 5-fold replicated (total of seventy experimental units). One to four individual plants of each species were collected on 18 July 2003 and transferred in coolers to the laboratory within two hours. Here individual plants were cut into fourteen pieces (ca. 1.5 g each piece, depending on the algae species). Two pieces of each species were weighed, and stored in a freezer (-20°C). The remaining twelve plant pieces were allocated to transparent plastic aquaria (2l), representing experimental units. Each plastic aquarium was individually supplied with a continuous flow of seawater from a reservoir directly connected to the seawater in the costal lagoon, thus reflecting the natural conditions inside the costal lagoon. In- and outflow of water were on opposite sides of individual aquaria, generating a unidirectional flow. Ten aquaria were used for each of the seven investigated species of algae. At the beginning of the screening experiment six pieces of each species were placed in each of the five control and the five direct-consumption aquaria. The experiment was subdivided into a (1) acclimation, (2) treatment and (3) recovery phase, each fourteen days long. In the acclimation phase, all algal pieces remained in the aquaria, to adjust to the cultivation conditions and recuperate from any putative predation in the field. At the end of the acclimation phase, from each aquarium one piece was transferred to the

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freezer (-20°C). Then, to start the treatment phase, grazers were added the five direct-consumption aquaria. Because previous observations revealed that the different algal species were consumed at different rates it was compensated this discrepancy by adding more or fewer herbivores. It was used five *B. reticulatum* for *U. lactuca*, one amphipod for *C. verticillatus*, *D. membranacea* and *F. vesiculosus* two amphipods for *C. tomentosum* and *S. coronopifolius* and three for *G. sesquipedale*. At the end of the treatment phase, three plant pieces were removed from each aquarium to serve as live alga item, artificial food item and growth control item, respectively to use in feeding preference assays (see 4.4. *Feeding preference assays with live algae*). Subsequently, to initiate the recovery phase, all grazers were removed from the aquaria to allow the algae to reduce their defence levels during a grazer-free two week period. The growth control item was put back in the aquaria. At the end of the recovery phase, the remaining three plant pieces were taken out of each aquarium and used in the same manner in feeding preference assays as described above after the treatment phase (Fig.6).

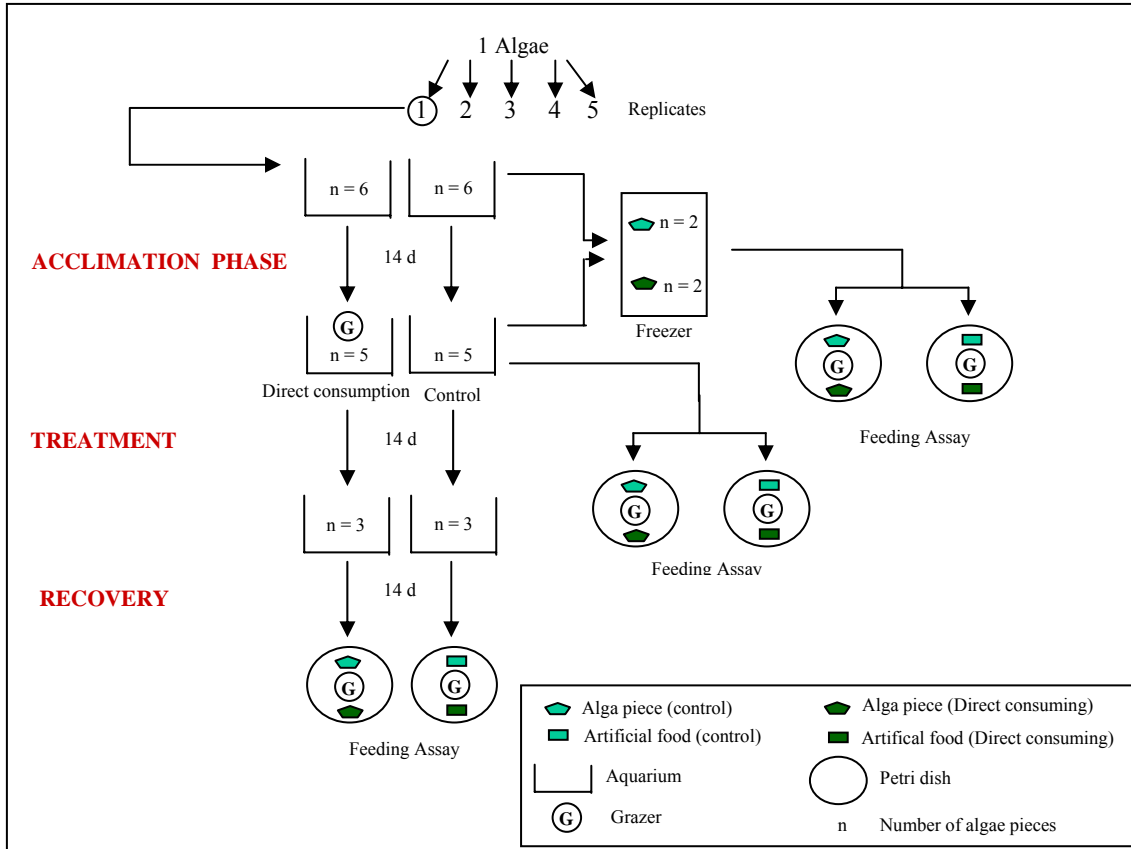


Figure 6 - Experimental set up for the first experiment (screening) with the three phases acclimation, treatment and recovery and the feeding assays. Example of a replicate: an alga species was cut in the beginning into 14 pieces and 12 were distributed on the aquariums. Two pieces were frozen immediately (in order to maintain all the defences that the alga could had on the field) and another two pieces were taken out of the aquaria in the end of the 14 days of the acclimation phase and also placed in the freezer for two days (with the aim of being in the same conditions as the previous ones). Afterwards the pieces were used in feeding preferences assays. In the beginning of the treatment phase the grazers were placed in the aquarium correspondent to the direct consumption treatment. In the end of this phase two more pieces were taken out for the feeding assays (together with two pieces to be used as growth control that were brought back into the aquaria after the feeding assays). The consumers were also removed, in order to reduce the defences during the Recovery phase. Finally, the remaining two pieces together with the 2 pieces of growth control were taken out of the aquaria and the feeding assays were accomplished.

**4.3. Induction Experiment Series - Second experiment (Water-borne cues detection)**

To detect whether water-borne cues released from grazers or grazed algae alone induce defences in other alga individuals, three algal species were used. The species were chosen taking into consideration the following criteria: (1) algae that appear to induce defences during the screening experiment, (2) the most resistant algae and (3) one of each group (red, green and brown). The experimental set-up was the same as in the first experiment (screening). The experiment was divided in four treatments and each one replicated 7 (*C. tomentosum* and *F. vesiculosus*) to 8-fold (*S. coronopifolius*) (together eighty eight experimental units). The four treatments were (Fig. 7):

- **Control** – without consumers.
  
- **Direct consumption** – algae were placed with consumers and therefore directly consumed.
  
- **Nearby grazed conspecifics** – additional pieces of the same species of the alga studied were placed in the same aquaria together with consumers. Additional pieces and the consumers were separated from the studied alga pieces with the help of a mesh, so that the examined pieces could detect any chemical cues coming out of the additional pieces and the consumers. This treatment should show whether the algae react to the nearby grazed conspecifics without being corroded.

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- **Non-grazing consumers** – as in the previous treatment, consumers were placed in the aquaria and separated from the studied alga pieces using a mesh. Thus the studied algae can detect any chemical cues transmitted from the consumers alone. This treatment should show whether the algae react to consumers without being corroded.

The algae for this experiment were collected on 13 September 2003. *C. tomentosum* and *F. vesiculodus* were collected at the Lagoon system of the Ria Formosa and *S. coronopifolius* at the rocky shore of São Rafael, and transferred in coolers within 2 h to the laboratory. As indicated by dashed line in Fig.7, a mesh (1mm) was fixed in the middle of all aquaria to separate consuming and non-consuming grazers in the upstream compartment (Fig. 7). Thus it could be tested whether the mere presence of nearby grazed conspecifics or non-grazing consumers is sufficient to release an inducible defence. In order to have similar conditions in all treatments, a mesh was fixed in every aquarium. The grazers used for the present experiment were the species *G. insensibles*, *G. fucicola* and *C. filosa*.

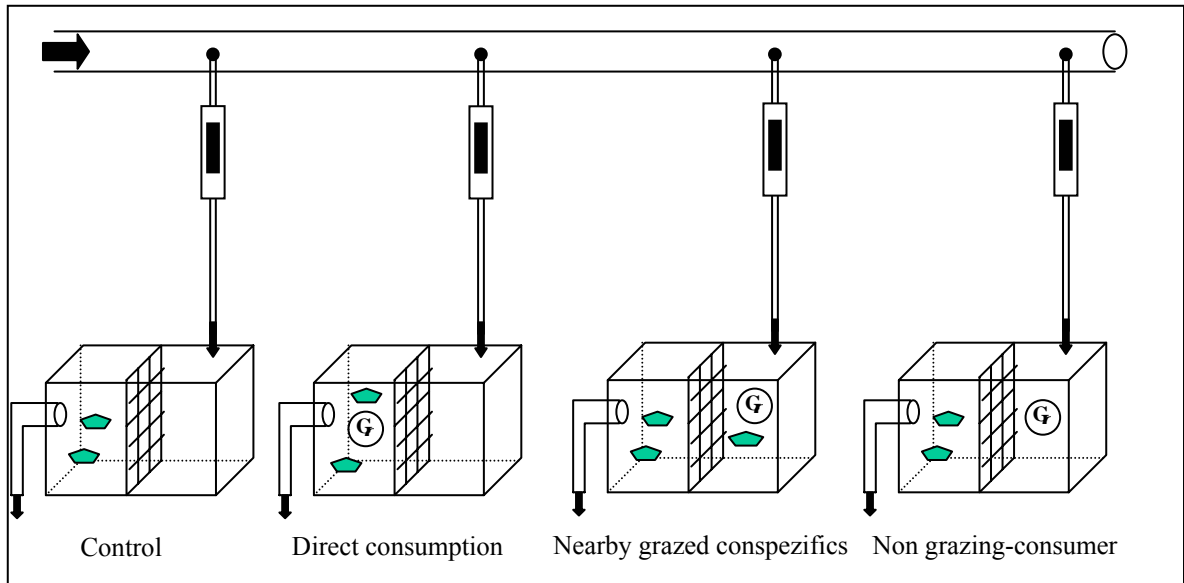


Figure 7 - Experimental set up (represents one replicate) for the water-borne cue detection experiment with the four treatments, the arrows show the waterflow.

#### 4.4. Feeding preference assays with live algae

The three algal pieces withdrawn after the treatment phase and recovery phase, respectively, were used in different choice feeding preference assays to determine whether formerly consumed plants were less palatable than control plants. The response variable was the biomass of differently treated algae consumed during the feeding assay. The first piece was offered as artificial food, the second piece as intact alga, the third piece served as growth control to adjust for autogenic biomass changes of intact algae pieces. The two feeding preference assays, with intact algae and artificial food, are accomplished to verify both the mechanical/chemical and purely chemical (lipophilic) aspects of the defence, respectively. The consumption rate is calculated by the difference of the wet weight of the piece before and after the feeding assay. This difference was corrected with the growth rate of the algae pieces, using the growth control piece (Taylor et al. 2002). The amount of intact live algae consumed was calculated as:

$$(T_{\text{start}} \times C_{\text{end}} / C_{\text{start}}) - T_{\text{end}}$$

Where  $T_{\text{start}}$  and  $T_{\text{end}}$  were pre-assay and post-assay wet masses of pieces exposed to grazing in the assay.  $C_{\text{start}}$  and  $C_{\text{end}}$  were pre-assay and post-assay wet weight mass of growth control pieces not exposed to grazers. In all feeding assays one control and one treated grazed plant piece were offered simultaneously in a Petri dish (15 cm Ø) to two to five consumers (mixture of *G. fucicola*, *G. insensibilis*, *C. filosa* depending on the alga tested) for two days. The water in Petri dishes was exchanged every 12h. Concerning the second experiments, all four pieces from the four treatments were

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offered simultaneously to the consumers. The herbivores used were not the same individuals used during the treatment phase in order to avoid physiological adjustments. Again the herbivores were not starved since this could have effects on its eating behaviour (Cronin and Hay 1996b). To distinguish food pieces within such a pair, 3 cm long threads of different colour were tied to them. The growth control pieces were placed in a separated Petri dish with the purpose of getting the alga growth without consumers.

The remaining plant piece was used to assess in choice feeding preference assays whether a lipophilic extract (see 5.5. *Feeding preference assays with diet containing non polar extracts*) of formerly consumed plants decreased the palatability of artificial food more than an equivalent extract of control plants. All the pieces used to make artificial food were frozen right after coming out the aquaria.

#### ***4.5. Feeding preference assays with diet containing non polar extracts***

To test whether the inducible defences belong to the non-polar secondary metabolites, artificial food preference assays with artificial food and lipophilic extracts were conducted. Treatment and control algae pieces were extracted for two days by 10 ml of dichloromethane (organic solvent responsible for the lipophilic extraction) at room temperature. Dried *Ulva lactuca*, a frequently used basis for artificial food, was ground in a blender. The quantity of *Ulva* powder was equivalent to 32% of the wet weight of each piece of alga. A mixture of 0.72 g of Agar in 10 ml of distilled water was heated to boiling in a microwave oven, allowed to cool for a few seconds, and then added to a mixture of powdered *Ulva*, 10 ml of dichloromethane extract and 16 ml of distilled water. The Dichloromethane was allowed to evaporate for 20 min prior the mixing, until the powder was dry. The proportions of the ingredients were calculated to closely match organic content and the natural concentration of non-polar metabolites. The combined mixture was poured over a mosquito mesh flattened between two layers of greaseproof paper with the help of two fibreglass panels (method adapted from Hay et al. 1994, 1998) (see Fig. 8). After cooling, the mesh was cut into squares of 15 x 15 cells and controls were identified by cutting a different corner for each treatment. The feeding assays were run like the feeding preference assays with the intact live alga. Instead of counting the squares consumed after the two days, all pieces were weighed before and after the feeding assay. The herbivores did not consume enough to empty the entire squares, scrapping off only the surface of the agar alga mixture.

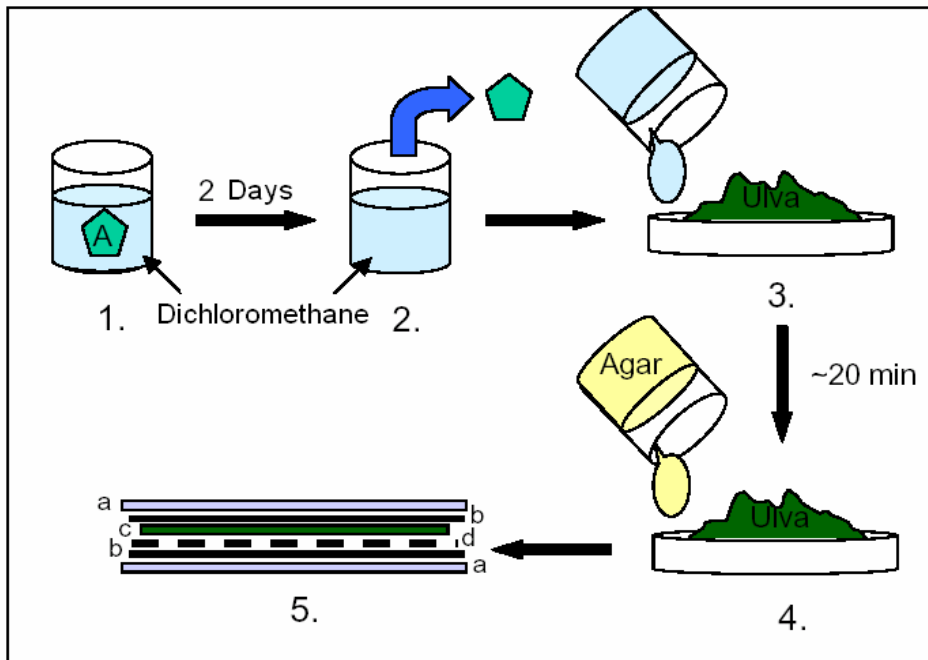


Figure 8 – Production of the artificial food: 1 – extraction of the alga in dichloromethane, 2 – piece of alga is removed, 3 – the dichloromethane (with the extraction) is mixed with *Ulva* powder and distilled water and then evaporate for 20 min, 4 - the *Ulva* powder (with the extraction) is mixed with the agar (previously heated with distilled water) and, 5 – the artificial food is flattened (a - fibreglass panels, b - greaseproof paper, c – *Ulva*-agar mixture, d – mosquito mesh).

*4.6. Statistical analysis*

Paired t-tests were used to analyse the data from the first experiment and the acclimation phase of the water-borne cues detection experiment. Prior to statistical analyses, the normal distribution of differences between control and grazed alga pieces was confirmed with the Kolmogorov–Smirnov test. Data showing heterogeneity of variances (Cochran’s test) were square root transformed. In order to compare the different treatments used in the cue - detection experiment during the treatment and recovery phases, a resampling method (Bärlocher 1999) (repeating 10000 times) was applied using a Monte Carlo analysis. Pairwise t-tests were used as post-hoc tests, for which no Bonferroni correction was calculated, if the probability of finding that the number of significant t-tests by chance was below 5% (Moran 2003).

# 5. Results

### **5.1. First experiment (screening)**

#### *5.1.1. Acclimation phase*

There were no significant differences in the consumption rates of intact algae when comparing pieces of algae frozen immediately after collecting and pieces of algae that were two weeks grazer-free (Table 2, Fig. 9). Regarding the artificial food, it was also not found any significant differences between freshly collected and acclimated algae of all the seven algae studied (Table 2, Fig. 9).

#### *5.1.2. Treatment phase*

After the treatment phase live control algae were generally more palatable than consumer-exposed conspecifics (Fig. 9). The brown seaweed *Fucus vesiculosus* was the only species showing a significant induction of defences (Table 2). On average, live control pieces of *Fucus* were 1.6 times more consumed than previously grazed conspecifics (paired t-test,  $t_4 = 4.83$ ,  $p < 0.05$ , Fig. 9). Artificial food made of *Fucus* control plants was on average 1.3 times more consumed than that of previously grazed plants (paired t-test,  $t_4 = 4.67$ ,  $p < 0.05$ , Fig 9). Again artificial food made from the other species showed no significant changes in palatability in the end of the treatment phase.

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	Algae	Intact-Algae		Artificial food	
		t	p	t	p
<b>Acclimation</b>	<i>Gelidium sesquipedale</i>	-2.44	n.s	-1.65	n.s
	<i>Sphaerococcus coronopifolius</i>	0.54	n.s	1.62	n.s
	<i>Cladostephus verticillatus</i>	11.15	n.s	-1.78	n.s
	<i>Ulva lactuca</i>	1.10	n.s	-0.53	n.s
	<i>Codium tomentosum</i>	0.99	n.s	1.71	n.s
	<i>Dictyopteris mambranacea</i>	10.71	n.s	0.48	n.s
	<i>Fucus vesiculosus</i>	-1.83	n.s	-0.64	n.s
	<b>Treatment</b>	<i>Gelidium sesquipedale</i>	-1.85	n.s.	7.27
<i>Sphaerococcus coronopifolius</i>		-1.34	n.s.	0.46	n.s.
<i>Cladostephus verticillatus</i>		-0.04	n.s.	1.39	n.s.
<i>Ulva lactuca</i>		-1.18	n.s.	-0.25	n.s.
<i>Codium tomentosum</i>		2.89	n.s.	-1.57	n.s.
<i>Dictyopteris mambranacea</i>		-1.58	n.s.	1.18	n.s.
<i>Fucus vesiculosus</i>		-4.83	<0.05	-4.67	<0.05
<b>Recovery</b>		<i>Gelidium sesquipedale</i>	-1.38	n.s.	-0.81
	<i>Sphaerococcus coronopifolius</i>	-0.36	n.s.	1.52	n.s.
	<i>Cladostephus verticillatus</i>	1.00	n.s.	-1.19	n.s.
	<i>Ulva lactuca</i>	-0.21	n.s.	-1.21	n.s.
	<i>Codium tomentosum</i>	1.46	n.s.	-1.20	n.s.
	<i>Dictyopteris membranacea</i>	0.20	n.s.	0.74	n.s.
	<i>Fucus vesiculosus</i>	-1.25	n.s.	-0.55	n.s.

Table 2: Screening experiment. Results of Student's t-test for 7 different algae species comparing consumption rates of algae pieces frozen immediately after collecting and pieces of algae that were two weeks grazer-free (acclimation phase). Comparison of consumption rates of previously grazed-exposed and non-exposed plants directly after 14 d long grazer exposure phase (treatment phase) and after an additional 14 d long grazer-free period (recovery phase). The analysis was performed with intact algae and a diet containing non polar extract (artificial food). n.s. – non significant

5.1.3. Recovery phase

The effect found after the treatment phase in live *Fucus* algae was no longer detectable at the end of the recovery phase, i.e., two weeks after grazing had ceased (Table 2, Fig. 9). The other algal species also showed no significant differences in the consumption rates in the end of this phase (Table 2, Fig. 9).

After the two grazer-free weeks, at the end of the recovery phase, the induced defence in *Fucus* had been reduced again and the palatability of artificial food from previously grazed individuals no longer differed from that made from the control plants (Table 2, Fig. 9). In the other species artificial food from previously treated and control individuals did not differ (Table 2, Fig. 9).

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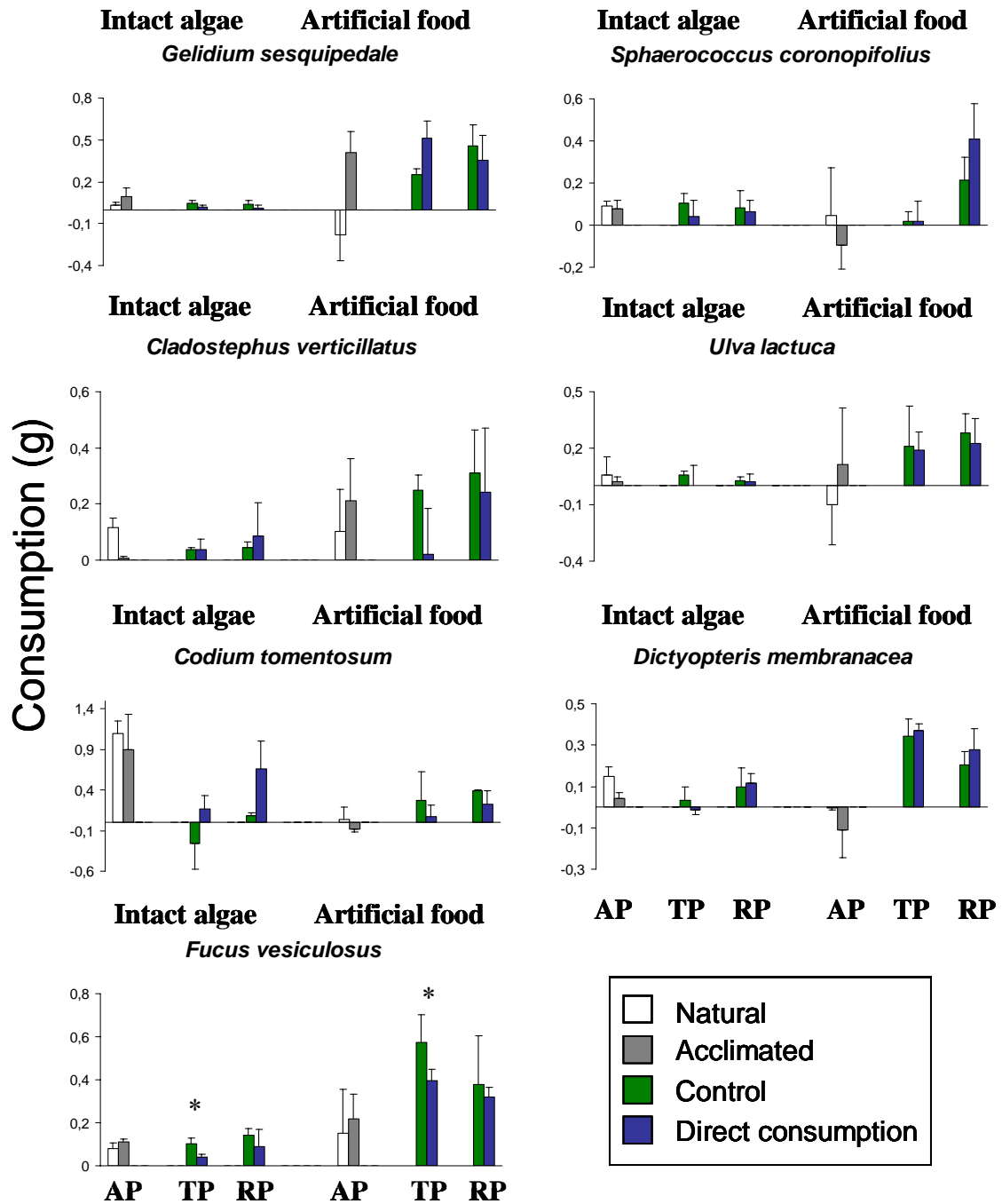


Figure 3 - Fig. 3: Screening experiment. Mean consumption [g] (average+SD) of 7 species of intact algae and artificial food after acclimation, treatment and recovery phase. AP- Acclimation Phase TR- Treatment Phase, RP- Recovery Phase. \* - significant results

## 5.2. Second experiment (water-borne cues detection)

### 5.2.1. *Sphaerococcus coronopifolius*

Palatability levels of live algae and artificial food of the red alga, *Sphaerococcus coronopifolius*, did not change significantly during acclimation phase (Table 3). After two weeks of grazer exposure, palatability levels in live *Sphaerococcus coronopifolius* plants exposed to different grazing cues were significantly reduced compared to non-exposed control pieces (Table 3). Post-hoc tests revealed that live control pieces of *S. coronopifolius* were significantly more preferred than conspecifics previously exposed to (1) grazers, and waterborne cues from (2) nearby grazed plants and from (3) a nearby non grazing consumer. Whether the herbivore was physically consuming the target individual or whether only a conspecific neighbour was attacked made no tangible difference with regard to defence induction in the target individual (minus 82% versus minus 84% palatability decrease). The mere presence of a non-feeding grazer, however, had a still significant but substantially weaker effect (minus 69%) on defence induction than the other 2 cues. At the end of the recovery phase, differences in palatability levels of live *S. coronopifolius* plants of different grazer exposures were no longer observed (Table 3, Fig. 10). Consumption rates of artificial food made of this alga were not significantly affected by different grazer exposures, neither after the treatment, nor after the recovery phase (Table 3).

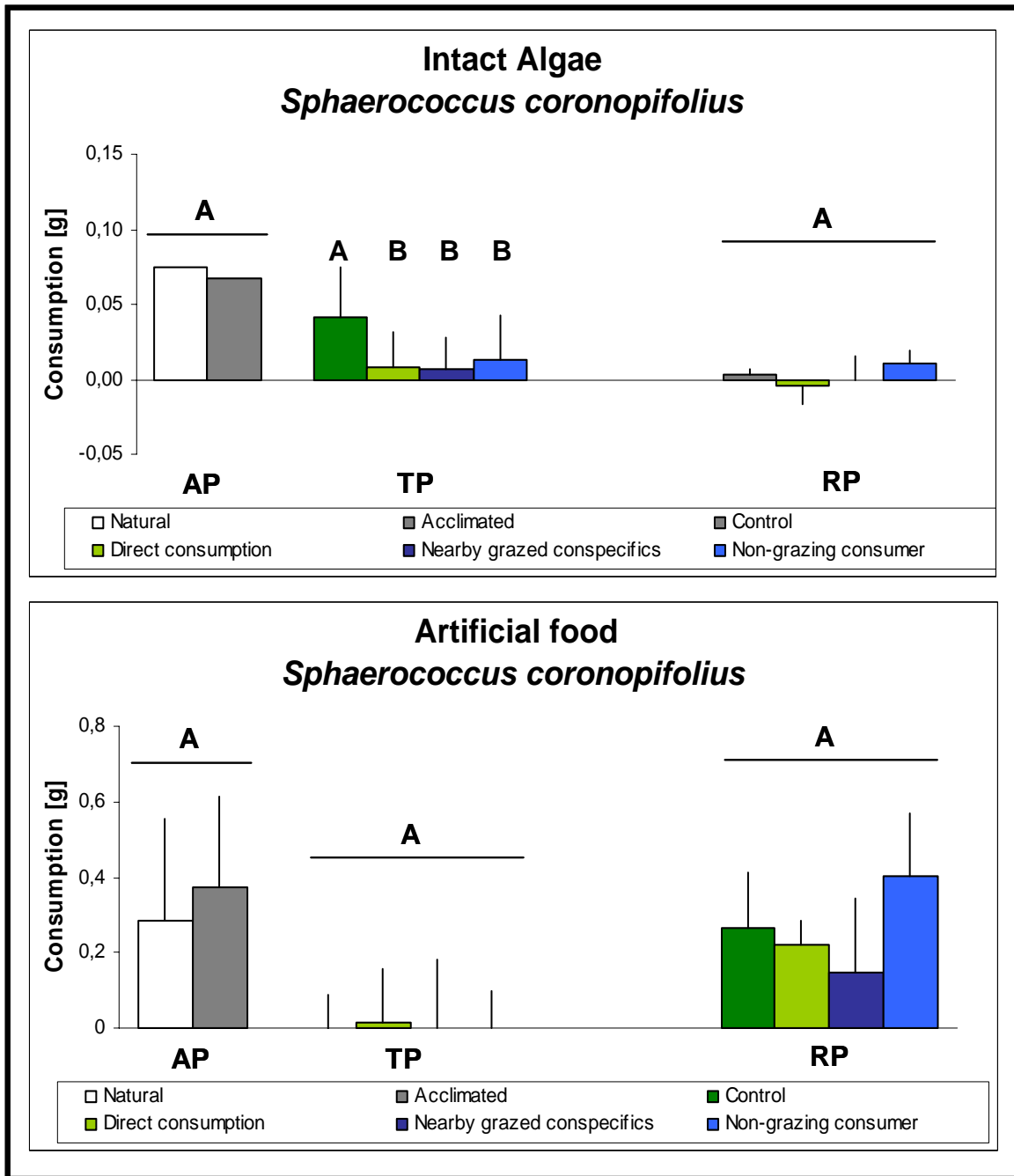


Figure 10 - Fig. 4: Water-borne cue induction experiment. Mean consumption [g] (average+SD) for *Sphaerococcus coronopifolius* after the acclimation (AP), treatment (TP) and recovery (RP) phases of intact algae and artificial food used in feeding assays. Feeding assays were conducted with previously incubated algal pieces, exposed to direct consumption, nearby grazed conspecifics and non grazing-consumers by three amphipods and control algae. Different letters show significant differences between the control and the treatment.

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Phase	Algae	Intact-Algae		Artificial food	
		t	p	t	p
<b>Acclimation</b>	<i>Sphaerococcus coronopifolius</i>	0.12	n.s.	-0.78	n.s.
	<i>Codium tomentosum</i>	-0.91	n.s.	-1.56	n.s.
	<i>Fucus vesiculosus</i>	-0.60	n.s.	-0.99	n.s.
<b>Treatment</b>	<i>Sphaerococcus coronopifolius</i>		<0.05		n.s.
	<i>Codium tomentosum</i>		n.s.		n.s.
	<i>Fucus vesiculosus</i>		<0.05		n.s.
<b>Recovery</b>	<i>Sphaerococcus coronopifolius</i>		n.s.		n.s.
	<i>Codium tomentosum</i>		n.s.		n.s.
	<i>Fucus vesiculosus</i>		<0.05		n.s.

Table 3: Water-borne cue detection experiment. Results of paired t-tests for the acclimation phase and the results of the Resampling/Monte Carlo analysis comparing consumption rates among four grazing treatments after acclimation, treatment and recovery phase using intact algae and diet containing non polar extract (artificial food). n.s. – non significant

### 5.2.2. *Fucus vesiculosus*

After the fourteen days of the acclimation there were no significant differences between the freshly collected and acclimated algae of the brown alga *Fucus vesiculosus*. Consumption rates between live control plants and *Fucus* pieces exposed to cues emitted by nearby grazed conspecifics and non-grazing consumers were significantly reduced by 97% and 52%, respectively (Table 3, Fig. 11). Surprisingly, the reduction of palatability of plants directly consumed was least and non-significantly reduced. Two weeks later, at the end of the recovery phase, live *Fucus* plants continued to show significantly different palatability levels among treatments. Control plants were more

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consumed than *Fucus* pieces previously exposed to grazers (minus 99%) and non-grazing consumers (minus 87%). The effect of neighbour grazing is still evident, but no longer significant. Surprisingly, for artificial food made of *Fucus* plants we were unable to detect any effects on palatability of the different grazing cues neither after the treatment nor after the recovery phase (Table 3, Fig. 11).

### 5.2.3. *Codium tomentosum*

Palatability levels of live algae and artificial food of the green alga, *Codium tomentosum*, did not change significantly during acclimation phase (Table 3). Live plants and artificial food made of *Codium* did not show any sign for defence induction when exposed to the different cues (Table 3, Fig. 12). After the fourteen days grazer-free it also showed no significant differences between all the treatments, for both the intact algae and artificial food (Table 3, Fig. 12).

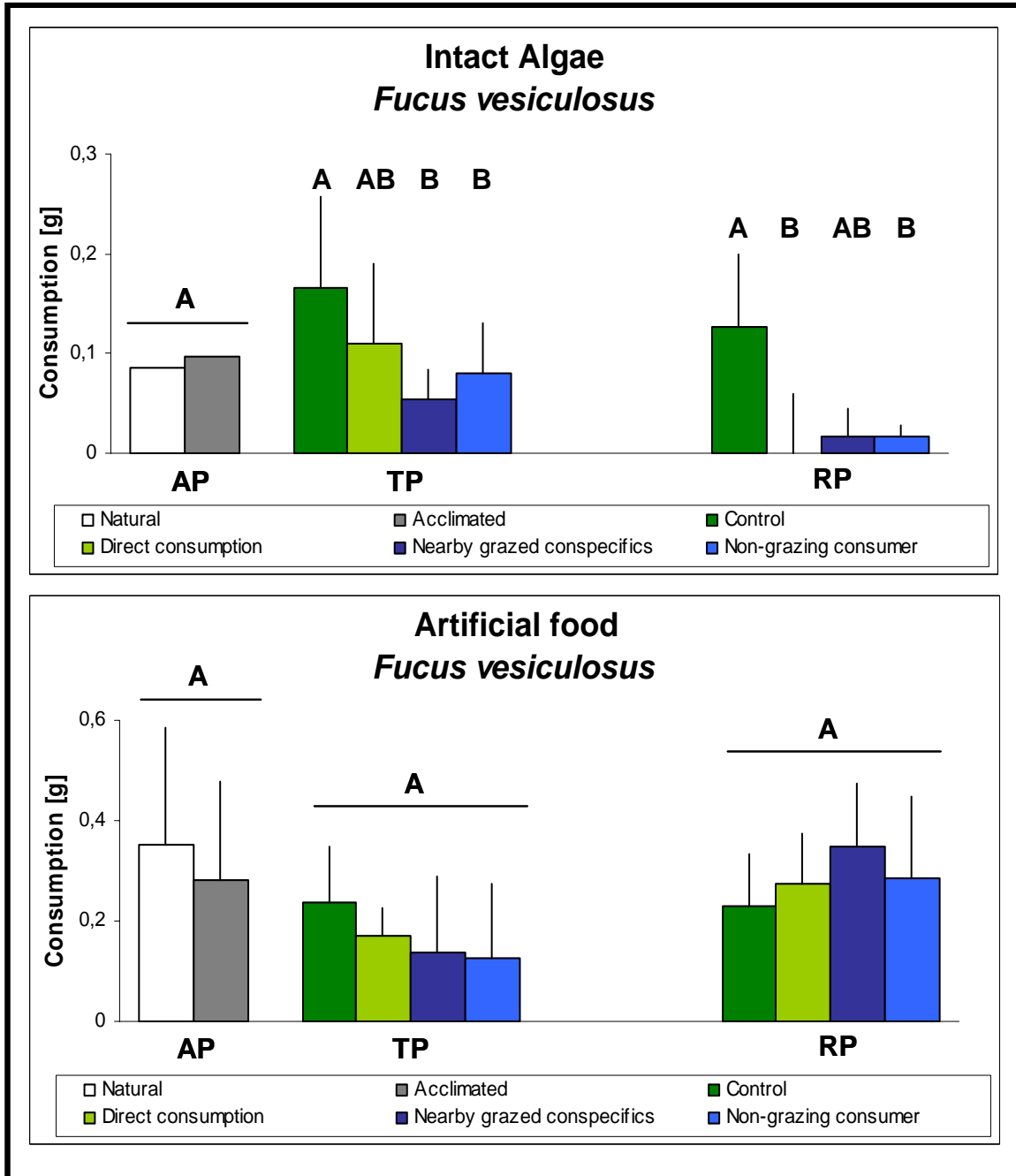


Figure 11 - Water-borne cue induction experiment. Mean consumption [g] (average+SD) for *Fucus vesiculosus* after the acclimation (AP), treatment (TP) and recovery (RP) phases of intact algae and artificial food used in feeding assays. Feeding assays were conducted with previously incubated algal pieces, exposed to direct consumption, nearby grazed conspecifics and non grazing-consumers by three amphipods and control algae. Different letters show significant differences between the control and the treatment.

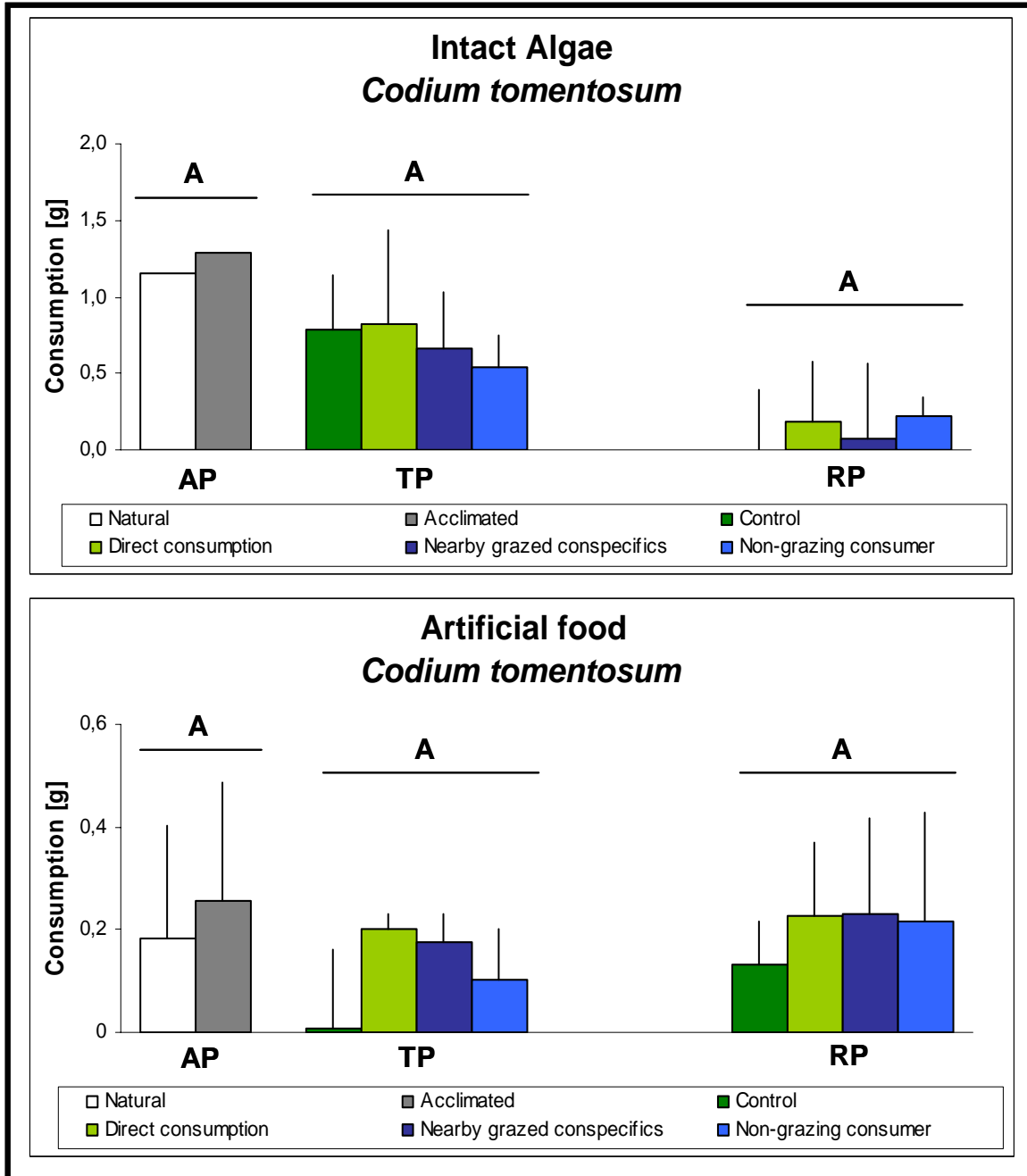


Figure 12 - Water-borne cue induction experiment. Mean consumption [g] (average+SD) for *Codium tomentosum* after the acclimation (AP), treatment (TP) and recovery (RP) phases of intact algae and artificial food used in feeding assays. Feeding assays were conducted with previously incubated algal pieces, exposed to direct consumption, nearby grazed conspecifics and non grazing-consumers by three amphipods and control algae. Different letters show significant differences between the control and the treatment.

## **6. Discussion**

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Generally, plants exposed to grazer cues tended to experience some reduction of palatability relative to control plants. During the two experiments, in two of the seven tested species these effects were significant. Palatability reduction can be interpreted as a response to grazer cues as induced defence in a broad sense. The brown seaweed *Fucus vesiculosus* induced defences when directly exposed to amphipods in the first experiment. In the second experiment both, *F. vesiculosus* and the red seaweed *Sphaerococcus coronopifolius* induced a defensive response to direct grazing as well as to water-borne cues from nearby grazed conspecifics and non-grazing amphipods.

### **6.1. First experiment (Screening)**

#### *6.1.2. Acclimation phase*

After a 14 days long grazer-free period, all acclimated algae showed no significant difference in palatability when compared to simultaneously collected, non-acclimated conspecifics. Therefore, no structural or chemical changes were observed that could have affected the grazers feeding behaviour. At least three explanations for this consistent pattern seem plausible: 1. the algae were exposed to any or a low herbivore pressure while still on the field, so that no defences were developed in order to be reduced at the end of the acclimation phase. They also could have a high defence level given the possibility of owning a constitutive defence. 2. It would be possible that the induced defence needs more than two weeks to reduce the current level of defence. 3. Possibly the preparation and cultivation procedures have a negative effect in the algae

palatability. This could be the case if the algae are subjected to high physiological stress (e.g. damaged by cutting) keeping the defence level high.

### 6.1.2. Treatment phase

The only species which in the screening experiments exhibited significantly lower palatability in grazer-exposed (= treated) compared to ungrazed (= control) plants was the brown seaweed *Fucus vesiculosus* (= *Fucus*) when offered as extracts embedded in artificial food. This demonstrates the ability of *Fucus* to induce repellent or toxic anti-herbivore metabolites within 14 days. A simultaneously induction of morphological defences can not be excluded since live control algae were also preferred over treated ones. However, it was not observed macroscopic morphological changes as reported from other studies, affecting e.g. growth form (Lewis et al. 1987) or growth of adventitious branches (Van Alstyne 1989). Less obvious morphological changes, e.g. tissue toughness or blade shape (Lowell et al. 1991, Ruuskanen and Back 1999, Taylor et al. 2002), were not controlled in the present experiment and can not be ruled out. Grazer mediated changes in the nutritive value represent still another possible explanation for the observed reduction of palatability (Van Alstyne et al. 2001), but no measures of nutrient concentrations were accomplished. A change in palatability of grazed *Fucus* may theoretically also occur if the consumers removed the more palatable parts of the alga, leaving less palatable portions untouched. However, this seems unlikely because amphipods consumed only a small fraction, i.e. <5%, of *Fucus* wet mass during the treatment phase. These results agree with previous studies that showed that different brown seaweed closely related to Fucales algae induced defences in

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marine habitats in response to herbivory (Cronin and Hay 1996a, Pavia and Toth 2000, Toth and Pavia 2000, Sotka et al. 2002, Taylor et al. 2002, Rhode et al. 2004). It corroborates in particular the studies of Rhode et al. (2004), where the isopod *Idotea baltica* induced defences in a *Fucus vesiculosus* population from the Baltic Sea. As far as it seems, this is the first time that two different populations of the same algal species were shown to induce defences stimulated by different grazer species in two independent experiments. The plasticity in the defence response to at least two grazer species indicates that this cosmopolitan brown alga may respond on demand to a broader taxonomic range of consumers. An induction of morphological defences has been described for other *Fucus* species. For example, *Fucus distichus* generates adventitious branches (mentioned above) in response to the attack of littorine snails (Van Alstyne 1989). Interestingly, significant discrimination among differently treated artificial food in the screening experiment and a similar but non-significant trend in the second experiment suggest that the defensive compounds are contained in the lipophilic fraction of secondary metabolites. But it may not be excluded that additional defence activities could be found among more polar metabolites as reported in earliest studies which suggested that phlorotannins may function as inducible defence compounds (Steinberg 1985, Van Alstyne 1988, Yates and Peckol 1993). The role of phlorotannins has been well studied in this context, but results are controversial. As pointed out by Hay (1996), these studies were lacking thorough testing hypotheses and that further investigations should focus on the relevance of lipophilic compounds. Algal palatability after induction of phlorotannins stimulated (Pavia et al. 1997), decreased, or did not affect (Pavia and Toth 2000) seaweed consumption by the mesograzers *Littorina obtusata* or *Idotea granulosa*. Also, even at elevated concentrations, phlorotannins lack

repulsive effects against at least some mesograzers, including amphipod species (Kubanek et al. 2004). Moreover, it was found that phlorotannins can act as feeding attractiveness instead of common deterrent to herbivores (Jormalainen et al. 2001). The rigorous bioassay guided study of Deal et al. (2003) revealed that *F. vesiculosus* compounds, which were repulsive to urchins, were neither within the phlorotannin- nor the DCM-soluble fraction. According to the present study, and contrary to Deal et al. (2003), an amphipod repulsive compound seems to occur primarily in the lipophilic fraction of the *Fucus*-chemistry.

### 6.1.3. Recovery phase

After the grazer-free recovery phase grazers did not discriminate anymore between treated and control algae. Apparently the defence in *Fucus* is not only inducible but also reducible. There was a succession in defensive traits, i.e. no difference in palatability after the acclimation phase, significant differences between treatment and control pieces after the treatment phase and then again no more response after the 14 grazer free days, which reflects that there were inducible cues. We can not say exactly when the level of the defences is reduced, but in this study we could detect that the relaxation took place in the 14 days of the recovery phase. Experiments of Peckol et al. (1996) with *Fucus* showed that there was a defence after three days and it was of relatively long term (more than two weeks). While defence reduction after cessation of a stimulus has been shown before (Hammerstrom et al. 1998), its finding is not really surprising. In a regulable defence system, induction on demand should be followed by reduction when the demand has passed. Hammerstrom et al. (1998) found a relaxation of defences in

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some kelp species, with many induced responses being relaxed in the day seven of the experiment, after clipping. Therefore, further experiments should be conducted with the aim of precise the period in which the defence level is maintained. Selective advantages of regulable defence could be the reduction of (1) autotoxicity (toxic products are not stored in the plant so the toxicity danger is reduced), (2) the putative costs of defences (costs are minimized while fitness is maximized, since resources can be more effectively used (Cronin 2001) and (3) the risk of exuded metabolites being 'misused' as cues by adapted consumers, parasites or epibionts.

The other six algae tested showed no effect of direct consumption on palatability in the screening experiment showing that no inducible defence can be proven. One possible explanation could be that these algae have constitutive defences or no defence at all. It is assumed that the green algae *Halimeda*, *Udotea* and *Caulerpa* have constitutive defences (Hay 1996). Hay and Steinberg (1992) suggest that herbivore specific defences can be developed. Rohde et al. (2004) showed that *Fucus vesiculosus* from the Baltic Sea induces defences against the isopod *Idotea baltica* when directly grazed and nearby grazed conspecifics. However, the gastropod *Littorina littorea* did not induce defences when directly grazed in *Fucus* showing that algal responses may be grazer specific. Therefore the algae studied might have defences that can be more effective to different herbivores, not used in this study. In order to determine this, more studies should be conducted using different species of herbivores. Another explanation for the lack of results could be a low herbivore pressure. Probably the number of grazers used in this experiment was not enough to induce defences. In addition, the defence can exhibit a seasonal dependence. This can be explained with differences of herbivore

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concentrations between the early and latter summer. If there is a rise of the herbivore number in the end of the summer probably the algae would develop anti-herbivore defences during this period. Also different life stages could show differences in anti-herbivore defence. Van Alstyne et al. (2001) showed that adult pieces of alga were preferred over juvenile pieces by some herbivores. Since the life stages were not determined in this study it is not possible make any statement. Furthermore we are confident that our sample size was high enough to detect any defences if they were present, given the power analyses conducted for these tests.

## 6.2. Second experiment (water-borne cues detection)

In the second experiment, the effects of different grazer cues were assessed. Induced changes of palatability were found in intact algae for two of the three species studied, while in extract-containing artificial food none.

### 6.2.1. *Codium tomentosum*

The green alga *Codium tomentosum* showed no induction of defences in any of the treatments of the second experiment. This supports the results obtained in the first experiment (screening), indicating that this alga has either no or constitutive defences as mentioned before.

### 6.2.2. *Fucus vesiculosus*

*Fucus* confirmed again to induce defences in this experiment, having the ability to induce with direct cues at different seasons. However, in this experiment the effect was only found after the recovery phase indicating a delayed response of the defences when directly grazed. It could be speculated that the delayed inductive response may result from a seasonally variable herbivore activity. For example, *Fucus* sensitivity to amphipod grazing may change seasonally, due to differential allocation of defensive compounds in response to seasonal performance for growth and reproduction. Second, seasonal differences in amphipod abundance are known to affect grazing pressure on algae (e.g. Worm 2000), but these data are missing for our study site. Finally, amphipod

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activities are temperature-dependent. Decreasing water temperature lowers grazing rates in molluscs, isopods, or urchins (Jonne et al. 2006). In the present study, temperature dropped an almost negligible 2° C between the July and the September experiments, making temperature-dependent effects on amphipod grazing unlikely. Seasonal differences in the inducibility of anti-herbivory defences were rarely investigated, but reported for the kelp *Ecklonia cava* (Molis et al. 2006). Here water temperature had been identical across seasons, suggesting that factors other than temperature drove changes in repulsive responses in *Ecklonia cava*.

In contrast to what happened in the screening experiment, no inducible defences were detected for the artificial food in any treatment. This may be due to changes in the identity of effective algal anti-herbivory compounds across seasons. Phenotypic plasticity is expected to lower the risk of consumer co-evolution (Agrawal and Karban 1999). If algae vary the kind of anti-herbivore compounds in combination with plastic responses, the risk of co-evolving consumers should be further lowered compared to inducing always the same defensive compound, as consumers need to adapt (1) to different defence molecules that are (2) not constantly present in the food. Additionally, *Fucus* induced defences when nearby conspecifics plants were grazed and when non-grazing consumers were around. This suggests an induction of anti-herbivory defences by water-borne cues, which has been already shown for three species of macroalgae, including *F. vesiculosus* (Toth and Pavia 2000, Rohde et al. 2004, Macaya et al. 2005). At the collection site, *Fucus* grows in dense stands. This facilitates the action of water-borne cues, which confer to *Fucus* conspecifics information about impending grazing (Rohde et al. 2004). The present study extends existing evidence about water-borne

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induction responses in *Fucus* driven by isopod grazers to amphipods. In contrast to the study by Rohde et al. (2004) dealing with isopod grazers, the present study indicates that water-borne info-chemicals released by the amphipod grazer alone, induced anti-herbivory defences in *Fucus*. Water-borne cues from mere grazer presence were generally considered as a deficient signal to induce stronger response to grazer attacks (Van Alstyne 1988, Toth and Pavia 2000, Rohde et al. 2004) rather than direct and nearby grazing. However, the present experiments reveal that *Fucus* may be sensitive to even non-grazing amphipods. This algal susceptibility to amphipods alone is surprising because amphipods use *Fucus* also as a habitat. Thus, *Fucus* specimens should permanently receive the inducing cue, which should lead to a permanent induction, i.e. a de facto constitutive defence. According to Karban et al. (1999), this quasi-constitutive response should minimise for the algae the selective advantage of using water-borne cues from non-grazing herbivores to induce anti-herbivory defences. Dissipation and dilution of cues emitted by the amphipods were possible much lower in our experimental units than in the natural environment. Possibly, this leads to cue concentrations, which in the field, would signal particularly high amphipod densities. Alternatively, *Fucus* may benefit from the ability to initiate defences as soon as the first signals of a strong grazer density are perceived, if amphipod abundances fluctuate sharply, resulting in sudden changes of grazing pressure. Testing this hypothesis requires identification of signal thresholds as well as seasonal monitoring of amphipod abundance. Finally, widespread induction response by *Fucus* should increase the number of distasteful conspecifics, which in turn may increase amphipod movements among algae, increasing amphipod visibility to their predators (Borell et al. 2004). Sotka et al. (2002), on the other hand, did not find an induction of resistance in

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*Sargassum filipendula* on waterborne cues, indicating that this species may not have physiological mechanisms to respond to waterborne cues. However, two genera of freshwater phytoplankton can change their behaviour or morphology in response to consumer-related water-borne cues (Tollrian and Harvell 1999), suggesting that this ability may be more common in the aquatic environment than hitherto thought.

### 6.2.3. *Sphaerococcus coronopifolius*

The red alga *Sphaerococcus coronopifolius* reacted strongly to all types of applied treatments. It induced defences when directly grazed, nearby conspecifics were grazed and when non-grazing consumers were present. In the screening experiment a similar tendency was observed but the effects had not been significant. This could mean that the reaction to direct consumption can be seasonal dependent. The first experiment started at the beginning of summer (July) and the second experiment at the end (September), and it was observed that the herbivores abundance rose at the end of the summer, suggesting a higher reaction at this time with an anti-herbivory defence. Until now, this is the first example of a red alga capable to induce defences on water-borne cues. It was already found induced defences for red algae, but only when they were directly grazed. Weidner et al. (2004) found inducible defences for the red alga *Pterocladia capillacea*, Ceh et al. (2005) for *Hypnea pannosa* and Díaz et al. (2006) for *Galaxaura diessingiana*, using an amphipod community dominated by *Elasmopus brasiliensis*, the amphipod *Cymadusa filosa* and the isopod *Paridotea rubra*, respectively. Most seaweeds, except some brown algae and siphonous green seaweeds, have poorly developed conducting tissues (Lobban and Harrison 1994). Therefore, water borne cues

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may be more important to these algae than to plants with a more developed internal transport system (Toth and Pavia 2000). For non-siphonous seaweeds like all Rhodophyta the capacity of reacting to water-borne cues may compensate for their deficiency in transmitting signals between different thallus areas.

Inducible defences were only found for the intact algae, with no effects for the artificial food, meaning that the defences can be either morphological or chemical (hydrophilic fraction) excluding the non polar compounds. Similar to what was showed for *Fucus*, this alga also can induce defences in the mere presence of the herbivore. Differences in palatability disappeared during the recovery phase, suggesting once more that there was a “switch off” mechanism and a reduction of defences. In contrast to what was observed in *Fucus*, the defence reduction in *Sphaerococcus* was completed within the fourteen day interval.

The ecological relevance of the effect of water-borne cues can be the information transported from one plant to another in a very fast way when they coexist in high stands. This function is very useful for relative fast moving mesoherbivores, as Rhode et al. (2004) found that water-borne cues are ineffective with slow moving grazers but effective with fast moving ones. In the present study we also used fast moving mesoherbivores, three species of amphipods, which supports the previous study referred.

# **7. Conclusion**

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With this study it has been shown that the brown alga *Fucus vesiculosus* can induce defences at different grazing exposures, confirming its capacity for inducing defences with new grazer species (*G. insensibilis*, *G. fucicola* and *C. filosa*) not only when directly consumed but also in the presence of conspecifics grazed plants and with mere presence of the grazer. Furthermore, it was found that the red alga (*Sphaerococcus coronopifolius*) may actually produce inducible responses when consumed directly, to the presence of actively feeding amphipods and also to the grazers alone. It was also found a seasonality of the production of defences in both algae that showed induction. The effectiveness of lipophilic compounds as well as speed and strength of inducible anti-herbivory defences were variable in time. This variation adds complexity to algae-mesoherbivore interactions, because inducible algae remain at least to a certain extent part of herbivore's diet rather than constitutively defended algae. Therefore, inducible defence could possibly advance stability in benthic systems similar to what is known from planktonic systems (Verschoor et al. 2004). The results show that inducible defences and inter-individual communication are more common than had been previously reported.

The present investigation was part of a modular project run in 9 different countries with the aim of identifying inducible anti-herbivore defences in macroalgae. Among the rhodophyta tested, inducible defences were found in *Hypnea pannosa* on the Kenyan coast (Ceh et al. 2005), *Galaxaura diessingiana* from the east coast of South Africa (Díaz et al. 2006), *Pterocladia capillacea* Atlantic coast of Brazil (Weidner et al. 2004), *Chondrus crispus* and *Polyides rotundus* on the west coast of Sweden (Langhamer pers. comm.), and *Mastocarpus stellatus* on the British North sea coast

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(Linnane pers. comm.). Thus, inducible anti-herbivore defences in red algae in particular and in macroalgae in general may be more common than hitherto thought.

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# APPENDIX I

EMAC V

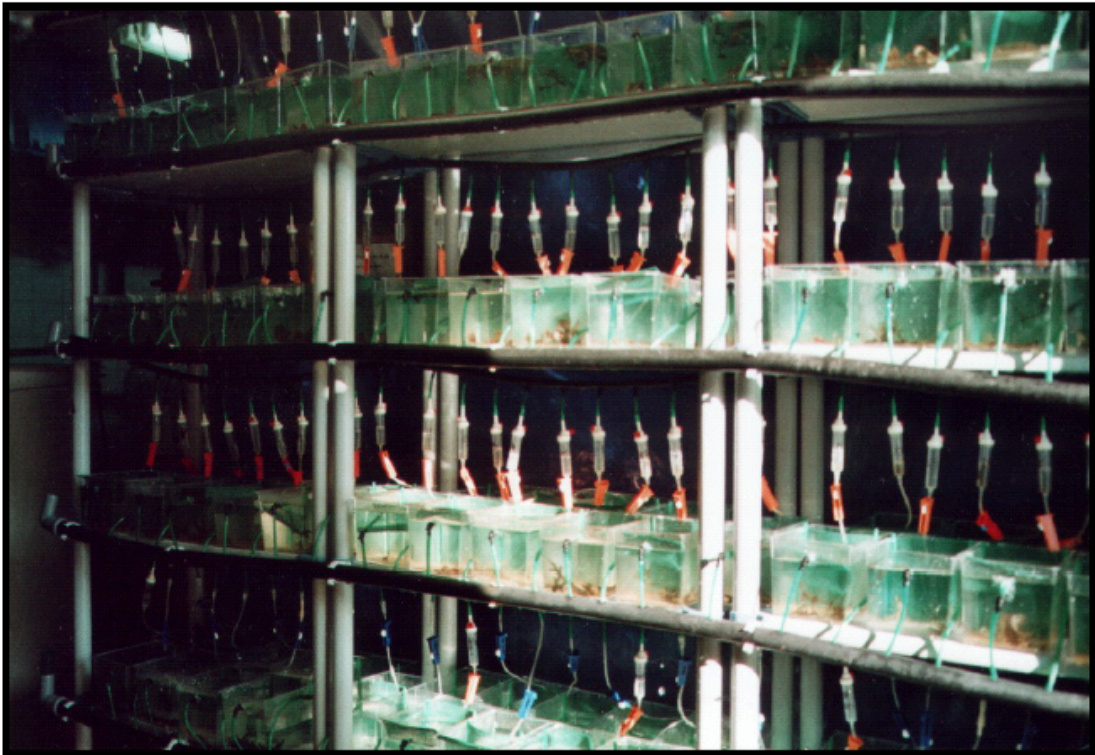


Figure 13 – Experimental set-up.

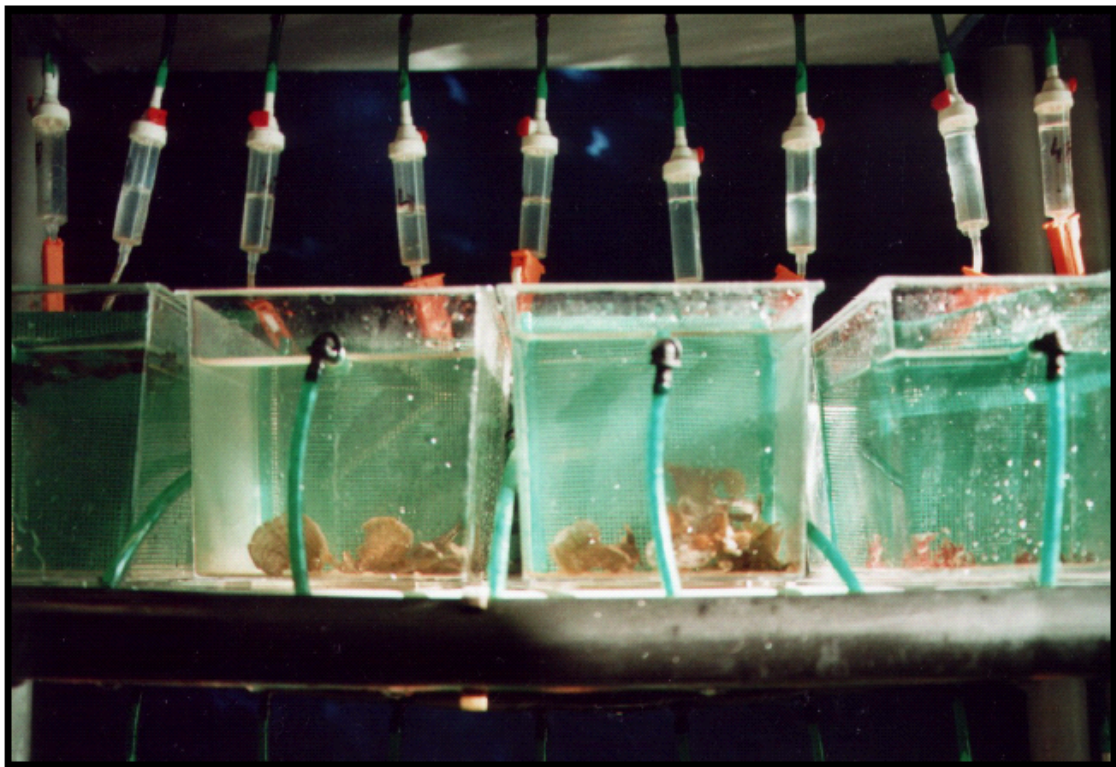


Figure 14 – Experimental set-up (detail).

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Figure 15 – Artificial food (diet containing non polar extracts) before cutting.



Figure 16 – Feeding preference assays (artificial food and intact algae)

# APPENDIX II

This work was promoted by an international training and research program

## **1. GAME - Global Approach by Modular Experiments**

### ***1.1. General description***

GAME is an international research program which is conducted by the Christian Albrechts Universität of Kiel. The project was developed by Professor Dr. Martin Whal (Leibniz Institute of Marine Sciences) in 2002 and granted by the Mercator Foundation GmbH.

Each year eight scholarships are given to students of foreign partner institutes and eight scholarships to German students, who are Masters or Honours students. In teams, the students run world-wide identical marine-ecological "twin experiments" in 8 different habitats. The addressed subject, as well as participating institutes vary among years, so a total of 20 institutes from all 5 continents are integrated in the program. Within each year experiments are staggered in two cycles between the southern and the northern hemispheres (Fig.1). Four foreign partner institutes participate in each cycle. An entire cycle lasts for 12 months, during which the students are directly involved over a period of 10 months. Each cycle is rationed into three phases (Fig.2).

*1.2. Investigation areas – First GAME-Cycle (2002-2004)*

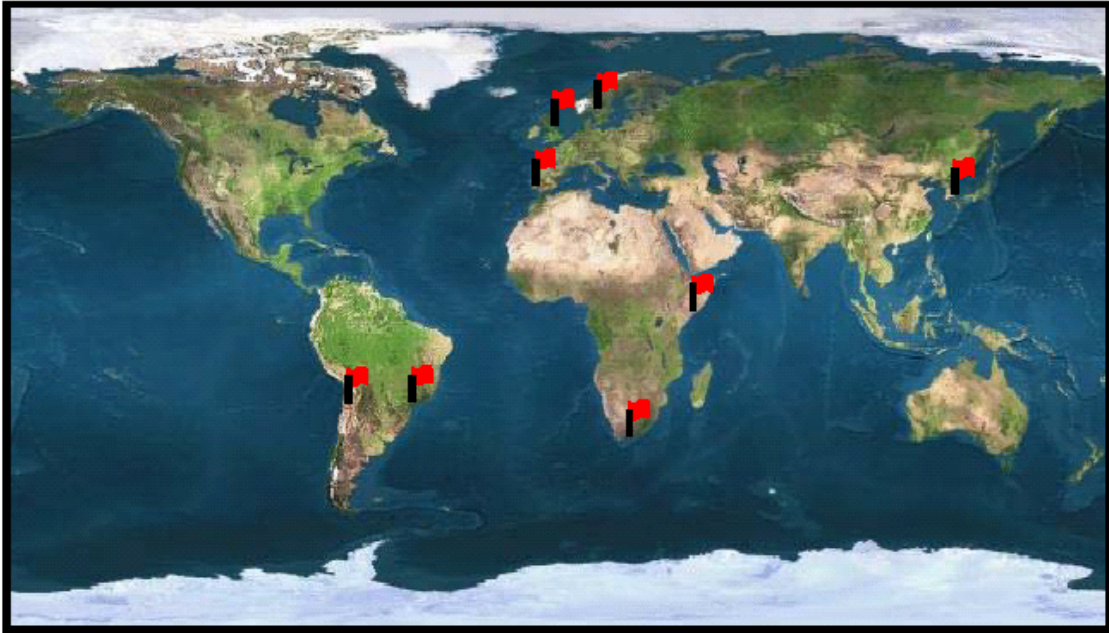



Figure 1 -  - Investigation areas of the Southern and Northern hemisphere.

Southern hemisphere:

- Brazil – Universidade Federal Fluminense
- Chile – Universidad Catolica del Norte
- Kenya – Kenya Marine and Fisheries Research Institute
- South Africa – Rhodes University

Northern hemisphere:

- England – Newcastle University
- Portugal – Universidade do Algarve
- South Korea – Sungkyunkwan University
- Sweden – Tjärnö Marine Biological Laboratory

### 1.3. Project – North hemisphere

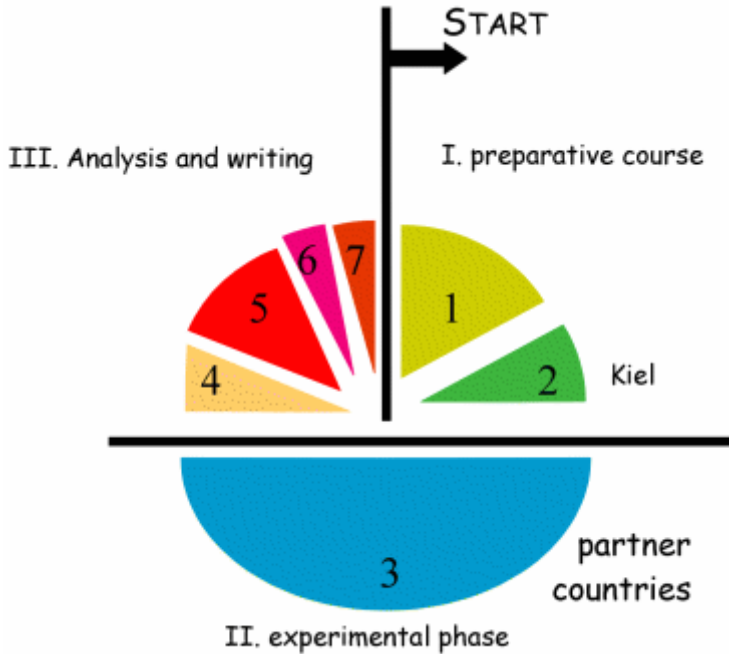


Figure 2 – Schedule of one GAME cycle, covering an entire year. Numbers refer to single segments (see text for details). Phase II takes place at the partner institutes abroad, phase I and III in Kiel (Leibniz Institute of Marine Sciences).

Phase I: This phase takes place entirely in Kiel and consists of a two month preparative and organizational section without students (see Fig. 2 no. 1), as well as a month-long preparative course (2) for all 8 participants of the cycle (4 German and 4 foreign students). During this course the students are trained in all the required theoretical and practical fields necessary to run the experiment abroad with confidence.

Phase II: Both, the native and the German student run the experiment abroad which is prepared during phase I in Kiel. These "twin experiments" are identical in their set-up, but use different sites, or in case of laboratory experiments, different organisms at the same site.

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Phase III: All 8 students from one cycle return to Kiel. Here, the generated data sets are analysed under the supervision of staff members of the department of "Experimental Ecology 2". In a first step, the required statistics is recapitulated and data from all students is compared, analysed and presented in graphical form during a two-week intensive course (4). During the following 7 weeks, each student writes his/her thesis (5). In the subsequent two weeks, manuscripts are written (6). Finally, it is planned to have one week of oral presentation of results by the students at some selected marine biology institutes in Northern Germany (7). In the Northern hemisphere cycle the following institutes were visited:

- Institut für Ostseeforschung, Warnemünde
- Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven
- Institut für Chemie und Biologie des Meeres, Oldenburg
- Universität Bremen

### ***1.4. Goals and topic***

The idea of GAME project is to answer a standing marine ecological question in a broad spatial and temporal scale through identical experiments that take place all over the world. The global replication intends to identify a general pattern despite the ecological variability. The topic of the first project questioned in what extent the macroalgae defences could be induced against herbivory. The experiments of the northern hemisphere groups were accomplished on laboratory conditions in England, Portugal, South Korea and Sweden.

# APPENDIX III

# Testing for the induction of anti-herbivory defences in four Portuguese macroalgae by direct and water-borne cues of grazing amphipods

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**Abstract** Herbivory is a key factor in regulating plant biomass, thereby driving ecosystem performance. Algae have developed multiple adaptations to cope with grazers, including morphological and chemical defences. In a series of experiments we investigated whether several species of macroalgae possess anti-herbivore defences and whether these could be regulated to demand, i.e. grazing events. The potential of direct grazing on defence induction was assessed for two brown (*Dictyopteria membranacea*, *Fucus vesiculosus*) and two red seaweeds (*Gelidium sesquipedale*, *Sphaerococcus coronopifolius*) from São Rafael and Ria Formosa, Portugal. Bioassays conducted with live algal pieces and agar-based food containing lipophilic algal extracts were used to detect changes in palatability after exposure to amphipod attacks (=treatment phase). *Fucus vesiculosus* was the only species significantly reducing palatability in response to direct amphipod-attacks. This pattern was observed in live *F. vesiculosus* pieces and agar-based food containing a lipophilic extract, suggesting that lipophilic compounds produced during the treatment phase were responsible for the repulsion of grazers. Water-borne

cues of grazed *F. vesiculosus* as well as non-grazing amphipods also reduced palatability of neighbouring conspecifics. However, this effect was only observed in live tissues of *F. vesiculosus*. This study is the first to show that amphipods, like isopods, are capable to induce anti-herbivory defences in *F. vesiculosus* and that a seasonally variable effectiveness of chemical defences might serve as a dynamic control in alga-herbivore interactions.

**Keywords** Amphipods · Anti-herbivory response · Induction · Macroalga-grazer interaction · Portugal · Seaweeds

## Introduction

Herbivory is a key factor for controlling biomass accrual and community structure of macroalgae (Hay and Fenical 1988; Hay 1991; Cyr and Pace 1993) and, thus, affecting a central ecosystem service. Depending on the food web structure of a system, meso-herbivores (Brawley 1992) may benefit from the presently observed overexploitation of predatory fish (Myers and Worm 2003), which would enhance their impact on macroalgae. Despite their low mobility and consumption rates relative to macro-herbivores (e.g. fishes), meso-herbivores (e.g. amphipods) due to their elevated numbers and more site-bound life histories may shape species composition of macroalgal communities (Brawley and Adey 1981; Duffy and Hay 2000).

In contrast to macro-herbivores, meso-herbivores may use algae as both food and habitat because they consume only part of an algal individual (Hay et al. 1987). This more lasting but less destructive association should influence algal responses to meso-herbivory. Algae can actively deter herbivores morphologically and/or chemi-

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cally (Hay 1996; Cronin 2001). To cope with a typically more sudden, more intense but less permanent attack by macro-herbivores such as fishes, a constitutive defence may be optimal. In contrast, meso-herbivores consume smaller portions of their food over longer time spans, which could favour the selection for defences that are adjusted to grazing pressure (inducible defence). In an evolutionary context, macroalgae displaying inducible anti-herbivory defences may have a selective advantage over constitutively defended algae for several reasons. First, the risk of self-intoxication is reduced because the production and storage of potentially harmful chemicals is minimised (Agrawal and Karban 1999). Second, algae with inducible defences represent a temporally and spatially variable (“moving”) target to herbivores, exacerbating herbivore adaptations to defensive algal traits (sensu Karban et al. 1999). Third, meso-herbivores move from induced to non-induced algae, which in turn exposes them more to predators (Borell et al. 2004).

An induction of anti-herbivory defences is well documented for vascular plants (Karbon and Baldwin 1997), but only limited evidence—essentially restricted to brown algae—exists for seaweeds. Present knowledge suggests that anti-herbivory defences in algae are a quite differential response. For instance, Pavia and Toth (2000) demonstrated that grazer identity matters as to whether defences were induced or not. Moreover, algal responses may be tissue-specific, when valuable algal parts, e.g. holdfasts, display constitutive and other tissues inducible defences (Taylor et al. 2002). Seasonal patterns of induction add further complexity to alga-grazer interactions (Molis et al. 2006). Induced defences were shown to be temporally and spatially variable. For example, the defence levels in *F. vesiculosus* were lowered to pre-grazed levels within 10 days (Hemmi et al. 2004). In addition, the repulsive response was not spreading to adjacent tissue (Hemmi et al. 2004). However, Toth and Pavia (2001) suggested that the information to defend may be propagated by water-borne cues within and between individual algae. To date only few studies have demonstrated the effects of water-borne cues in the context of inducing anti-herbivory defences in macroalgae (Toth and Pavia 2000; Rohde et al. 2004; Macaya et al. 2005). Knowledge on whether effective water-borne cues stem from the grazer or the alga is extremely scarce. Furthermore, little is known about the identity of chemical compounds that are induced during anti-herbivory responses.

The aim of this study was to test whether (1) direct grazer attacks induce defences in selected red and brown seaweeds, (2) water-borne cues from either conspecifics and/or grazers could induce anti-herbivory defences, and (3) the effects of the water borne cues might decrease, after grazing stopped.

## Materials and methods

### Collection sites and organisms

All algae and herbivores were collected at the intertidal rocky shores of São Rafael, Albufeira (37°05′N, 8°15′W) and the lagoon-system Ria Formosa (37°1′N, 7°55′W), Portugal. To ensure the ecological relevance of the research, study algae were selected by regional abundance and their supposed role as habitat engineers (large, bushy, perennial), and relevant herbivores were selected after assessing feeding preferences in pilot studies (data not shown). As a result, we chose two species each of red (*Gelidium sesquipedale* and *Sphaerococcus coronopifolius*) and brown seaweeds (*Dictyopteris membranacea* and *Fucus vesiculosus*). As herbivores, we selected an assemblage of three amphipod species (*Gammarus insensibilis*, *Gamarella fucicola*, and *Cymadusa filosa*) naturally associated with and feeding on the selected macroalgae at both collection sites. Hereafter algae are referred to by their generic names and herbivores as amphipods.

### Experimental set-up and design

We conducted two induction experiments, one in July and one in September 2003. These were divided into either two (acclimation and treatment, July experiment) or three (additional recovery, September experiment) 2-week phases. First, the effects of herbivore attack on the induction of anti-herbivory defences in live algal pieces were assessed for all four selected species of macroalgae. Treatment effects, i.e. change in palatability, were identified by feeding assays, where after the treatment phase the consumers could choose between a grazed and a non-grazed algal piece. To test whether an observed induction was based on non-polar secondary metabolites, we conducted feeding assays with artificial food containing broad-spectrum DCM extracts. The effects of water-borne cues were assayed in the September experiment with only *Fucus*.

For the July experiment, five specimens of each algal species ( $n = 5$ ) were collected and transferred in a cooler to the Marine Laboratory of Ramalhete, Faro, Portugal, within 2 h after collection on 18 July 2003. On the same day, all macroscopic epibionts were gently removed from all algae with a sponge. Six pieces (ca. 3 g per piece) of comparable tissue type were cut from each specimen. These six pieces were distributed at random between one control and one treatment aquarium (experimental units EUs), resulting in a total of 40 EUs (4 species  $\times$  5 individuals  $\times$  2 treatment levels) containing three genetically identical thallus pieces each. Each transparent plastic aquarium (2 l volume) was individually supplied with a continuous, unidirectional flow of seawater from a

reservoir directly connected to the coastal lagoon next to the marine station.

At the start of the July experiment, algal pieces remained for 14 days without consumers (acclimation phase) to adjust to cultivation conditions and to equalise possible differences in defensive traits, which may have been acquired by unknown grazing histories in the field. After acclimation, one (for *Fucus* and *Dictyopteris*), two (for *Sphaerococcus*), or three (for *Gelidium*) amphipod individuals were randomly drawn from the three-species mixture and added to each treatment EU and allowed to consume the macroalgae for 14 days, while no grazers were added to the control EUs (treatment phase). Thus individual study algae were exposed to one to three grazer species, but averaged over all replicates for a given algal species, the consumption quantified was due to the naturally occurring mixture of the three-amphipod species. Working with different consumer species within one treatment increases the scatter of results but reflects what is happening in the field. Grazer densities varied among algal species according to observed densities in the field in order to keep grazing pressure in our experiment comparable to natural levels of herbivory. At the end of the treatment phase, grazers were removed. The palatability of live algal pieces, and, when appropriate, artificial food was assessed in choice feeding assays (see below).

For the September experiment, seven *Fucus* specimens ( $n = 7$ ) were collected in the Ria Formosa lagoon on 13 September 2003. All EUs were vertically divided by a mesh (1 mm mesh size) into equally sized up- and downstream compartments. Twenty pieces of comparable tissue-type were cut from single plants and five pieces each randomly allocated to the downstream compartments of four EUs at the beginning of the acclimation phase (7 plants  $\times$  4 treatments = 28 EUs total). Four different treatment levels were allocated to the EUs: (1) addition of amphipods to the downstream compartment (test for direct grazer attack), (2) addition of amphipods and *Fucus* pieces to the upstream compartment (test for water-borne cues from nearby grazed conspecific algae and/or grazing amphipods), (3) addition of amphipods to the upstream compartment (test for water-borne cues from non-grazing amphipods), and (4) no addition of amphipods or algae to the upstream compartment (control). At the end of the treatment phase, all herbivores were removed from the EUs as well as all *Fucus* pieces from the upstream compartments. Moreover, 2 algal pieces were randomly withdrawn from the downstream compartment of each EU and used for either multi-choice feeding assays of live pieces or agar-based food containing lipophilic *Fucus* extracts, respectively. Two of the other three algal pieces remained in the EUs for another 14 days (recovery phase). The third piece was transferred during the assay period into a sepa-

rate, amphipod-free Petri dish (see next paragraph) to measure autogenic changes in wet mass and returned to EUs after assays were terminated. At the end of the recovery phase, the same algal piece was used again to measure autogenic changes in wet mass during assays. The remaining two algal pieces were withdrawn from EUs to assess their palatability levels in multi-choice feeding assays of either live pieces or agar-based food (testing for reduction of the defence after treatment).

#### Feeding preference assays

Two-way choice (July experiment) or multiple-choice (September experiment) feeding assays compared the relative palatability of differently treated algal pieces. In the July experiment, one live piece of a control and a grazed alga were offered to amphipods simultaneously in a Petri dish (15 cm Ø) for 4 days. The water in Petri dishes was exchanged every 12 h. In the September experiment, 4 differently treated algal pieces each (see above) was offered in the same Petri dish. For distinction during the feeding assays, algal pieces were coded by coloured threads. In the multiple-choice assay with artificial food, the pellets were individualized by different incision patterns. Different amphipod individuals were used in the treatment phase and in subsequent feeding assays to avoid consumer adaptations. Wet mass of assayed algal pieces was measured to the nearest 0.001 g at the beginning and end of feeding assays. Moreover, the wet mass change of a control algal piece from the same treatment as the assayed alga was used to correct consumption rates for non-feeding-related autogenic changes in wet mass, according to the Peterson and Renaud (1989) formula:

$$\text{consumption} = T_{\text{start}} \times (C_{\text{end}}/C_{\text{start}}) - T_{\text{end}}$$

where  $T_{\text{start}}$  and  $T_{\text{end}}$  represent pre- and post-assay wet mass of pieces of an assayed alga, respectively, and  $C_{\text{start}}$  and  $C_{\text{end}}$  represent pre- and post-assay wet mass of autogenic control pieces, respectively.

#### Preparation of artificial food

To determine whether the induced defences were due to secondary metabolites (activated or induced only for defensive purposes), feeding assays with lipophilic algal-extracts were performed for those algae that showed significant responses in assays using live algal pieces. Algal pieces were submerged in 10 ml of dichloromethane for 2 day at room temperature. Subsequently, the dichloromethane supernatant was poured onto *Ulva lactuca* powder and allowed to evaporate. In this manner, the *Ulva* powder was coated by the non-polar algal compounds. *Ulva* has

been used previously as a standard food, palatable to most herbivores, in assays using algal extracts (e.g. Deal et al. 2003). A mixture of 0.72 g Agar and 10 ml distilled water was boiled, allowed to cool to ca. 40°C, mixed with the algal compound-coated *Ulva* powder, and poured over a mosquito mesh flattened between two fibreglass panels (method adapted from Hay et al. 1994). After cooling, a 15 × 15 mm<sup>2</sup> section was cut from each pellet and used in feeding assays.

### Statistical analysis

Consumption rates from choice feeding assays (July experiment) were analysed by two-tailed paired *t*-tests. Prior to statistical analyses, the normal distribution of differences between control and grazed alga pieces was confirmed with the Kolmogorov–Smirnov test. Consumption rates from multiple-choice feeding assays (September experiment only) were analysed by resampling without replacement, using a Monte Carlo analysis with 10,000 permutations (Bärlocher 1999). Pairwise *t*-tests were used as post-hoc tests, for which no Bonferroni correction was calculated, if the probability of finding that the number of significant *t*-tests by chance was below 5% (Moran 2003).

## Results

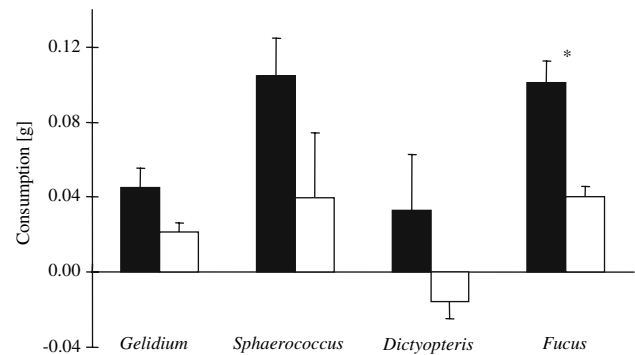
### July experiment

At the end of the treatment phase, live specimens of *Gelidium*, *Sphaerococcus*, and *Dictyopteris* showed a non-significant trend of higher palatability in control pieces than in amphipod-attacked pieces (all paired *t*-test:  $t_4 \leq 1.85$ ,  $P > 0.05$ ). Only for live *Fucus* pieces, amphipod consumption of control pieces was significantly higher (by 60% on average) than consumption of amphipod-attacked pieces (paired *t*-test:  $t_4 = 4.83$ ,  $P = 0.008$ , Fig. 1). This pattern was also detected when agar-based food containing lipophilic *Fucus*-extracts was used. Control pieces were significantly preferred by 31% over agar-based food containing lipophilic extracts from amphipod-attacked conspecifics (paired *t*-test:  $t_4 = 4.67$ ,  $P = 0.01$ , Fig. 2).

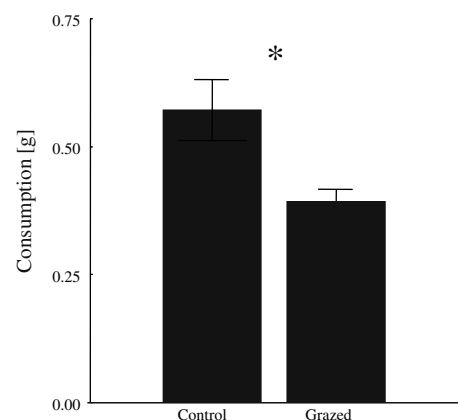
### September experiment

The experiment was performed with *Fucus*, the only species that had shown a significant reduction in palatability in response to amphipod attacks.

1. Live *Fucus*: At the end of the 2-week treatment phase, amphipod consumption was significantly different between differently treated *Fucus* pieces (resampling,

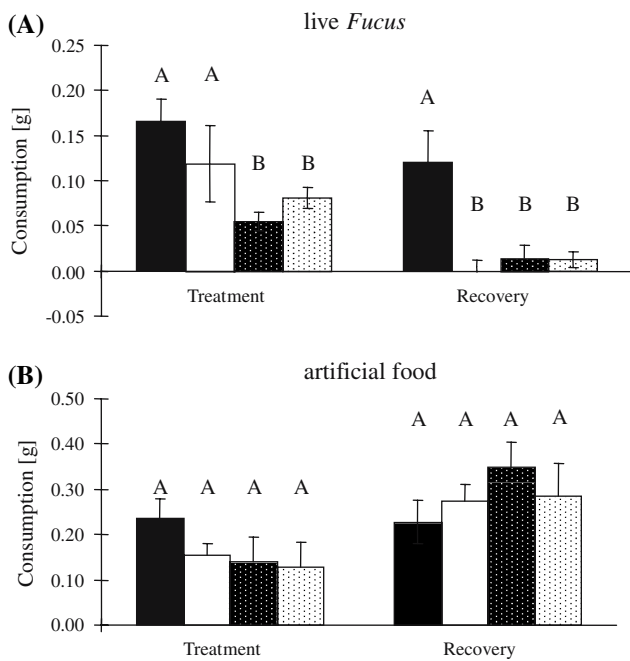


**Fig. 1** Mean (+SE) amphipod-consumption on live pieces of different alga species during 4-day long feeding assays ( $n = 5$ ) at the end of the treatment phase (see ‘‘Materials and methods’’ for details). Black bars controls, open bars directly amphipod attacked, \* significantly different



**Fig. 2** Mean (+SE) amphipod-consumption of artificial *Fucus vesiculosus* food during 4-day long feeding assays ( $n = 5$ ) at the end of the treatment phase (see ‘‘Materials and methods’’ for details). \* Significantly different

- $P = 0.019$ ). The palatability of individuals exposed to water-borne cues from nearby amphipod-grazed conspecifics and/or grazing amphipods, as well as to non-grazing amphipods, was significantly lower than the palatability of control pieces, while the palatability of directly grazed *Fucus* pieces was non-significantly decreased (Fig. 3a). Control pieces were on average 48% more palatable than grazed pieces. At the end of recovery phase, the palatability of *Fucus* controls increased even more. Control pieces were on average a significant 91% more consumed than *Fucus* pieces exposed to the other three treatments (resampling  $P = 0.050$ ).
2. Agar based food containing lipophilic *Fucus*-extracts. At the end of both treatment and recovery phase, amphipod consumption was not significantly affected by grazing treatments (resampling: treatment phase  $P = 0.137$ , recovery phase  $P = 0.561$ ; Fig. 3b).



**Fig. 3** Mean (+SE) amphipod-consumption on *Fucus vesiculosus* during 4-day long feeding assays ( $n = 7$ ) at the end of the treatment and recovery phase (see “Materials and methods” for details) for **a** live algae and **b** agar-based food containing lipophilic *Fucus*-extracts. Treatments sharing the same letter are not significantly different. Black bars control, open bars directly amphipod attacked, white dotted bars waterborne cues from nearby-grazed conspecifics, black dotted bars waterborne cues from non-grazing amphipods

## Discussion

In all algae species tested in the July experiment, control pieces were preferred over grazed pieces, but this was statistically significant only for *Fucus*. Also in the case of assays using artificial food containing lipophilic *Fucus*-extracts, control pieces were preferred over directly grazer-exposed pieces. As shown in the September experiment, exposure of *Fucus* to water-borne cues from nearby compartments containing either amphipods grazing on *Fucus* or amphipods alone, also reduced the palatability of live alga pieces but not that of artificial food containing a respective alga extract. A change in palatability of grazed *Fucus* may theoretically also occur if the consumers removed the more palatable parts of the alga, leaving less palatable portions untouched. However, this seems unlikely because amphipods consumed only a small fraction, i.e. <5%, of *Fucus* wet mass during the treatment phase. In addition, control pieces of *Fucus* continued to be more palatable than grazed pieces even after grazing had ceased for 14 days and new tissues had been formed. Alternatively, this change in palatability suggests that direct amphipod grazing induced an anti-herbivore response of morphological and/or chemical origin. In addition, the significant reduction in

consumption of artificial food containing extracts of amphipod-attacked *Fucus*, hints at an induced production or mobilization of lipophilic, i.e. non-phlorotannin, compounds with anti-herbivory effects. An induction of chemical anti-herbivory compounds in brown seaweeds has been reported by several studies (e.g. Rohde et al. 2004). The role of phlorotannins has been well studied in this context, but results are controversial. Algal palatability after induction of phlorotannins stimulated (Pavia et al. 1997), decreased, or did not affect (Pavia and Toth 2000) seaweed consumption by the mesograzers *Littorina obtusata* or *Idotea granulosa*. Also, even at elevated concentrations, phlorotannins lack repulsive effects against at least some mesograzers, including amphipod species (Kubanek et al. 2004). The rigorous bioassay guided study of Deal et al. (2003) revealed that *F. vesiculosus* compounds, which were repulsive to urchins, were neither within the phlorotannin- nor the DCM-soluble fraction. According to the present study, and contrary to Deal et al. (2003), an amphipod repulsive compound seems to occur primarily in the lipophilic fraction of the *Fucus*-chemistry. In addition to chemical defences, amphipods may induce morphological anti-herbivory defences in *Fucus*. An induction of morphological defences has been described for other *Fucus* species. For example, *F. distichus* generates adventitious branches in response to the attack of littorine snails (van Alstyne 1989). We did not observe the production of new tissues, but cannot rule out that *Fucus* induced less obvious morphological changes, e.g. tissue toughness or blade shape (Lowell et al. 1991; Ruuskanen and Back 1999), for which no measurements were taken. But morphological defences—if induced—were in any case not the only ones, since a (slightly smaller) anti-herbivory activity was also found in the extracts.

Inductive patterns were altered in two ways when experiments were repeated later in the year. On the one hand, anti-herbivory defences were detected with some delay in live *Fucus*, i.e. only at the end of the recovery phase, and on the other hand effects were not detected with artificial food containing lipophilic *Fucus*-extracts. It could be speculated that the delayed inductive response may result from a seasonally variable herbivore activity. For example, *Fucus* sensitivity to amphipod grazing may change seasonally, due to differential allocation of defensive compounds in response to seasonal performance for growth and reproduction. Second, seasonal differences in amphipod abundance are known to affect grazing pressure on algae (e.g. Worm 2000), but these data are missing for our study site. Finally, amphipod activities are temperature-dependent. Decreasing water temperature lowers grazing rates in molluscs, isopods, or urchins (Jonne et al. 2006). In the present study, temperature dropped an almost negligible 2°C between the July and the September

experiments, making temperature-dependent effects on amphipod grazing unlikely. Seasonal differences in the inducibility of anti-herbivory defences were rarely investigated, but reported for the kelp *Ecklonia cava* (Molis et al. 2006). Here water temperature had been identical across seasons, suggesting that factors other than temperature drove changes in repulsive responses in *Ecklonia cava*. The second deviation from the July experiment, i.e. lacking effects in assays using artificial food containing lipophilic *Fucus*-extracts, is interesting. This may be due to changes in the identity of effective algal anti-herbivory compounds across seasons. Phenotypic plasticity is expected to lower the risk of consumer co-evolution (Agrawal and Karban 1999). If algae vary the kind of anti-herbivore compounds in combination with plastic responses, the risk of co-evolving consumers should be further lowered compared to inducing always the same defensive compound, as consumers need to adapt (1) to different defence molecules that are (2) not constantly present in the food.

Amphipods consumed significantly less tissue from *Fucus*-specimens located down-stream of nearby grazed conspecifics than from control algae. This suggests an induction of anti-herbivory defences by water-borne cues, which has been already shown for three species of macroalgae, including *F. vesiculosus* (Pavia and Toth 2000; Rohde et al. 2004; Macaya et al. 2005). At the collection site, *Fucus* grows in dense stands. This facilitates the action of water-borne cues, which confer to *Fucus* conspecifics information about impending grazing (Rohde et al. 2004). Our study extends existing evidence about water-borne induction responses in *Fucus* driven by isopod grazers to amphipods. In contrast to the study by Rohde et al. (2004) dealing with isopod grazers, the present study indicates that water-borne info-chemicals released by the amphipod grazer alone, induced anti-herbivory defences in *Fucus*. Water-borne cues from mere grazer presence were generally considered as a deficient signal to induce stronger response to grazer attacks (van Alstyne 1988; Toth and Pavia 2000; Rohde et al. 2004) rather than direct and nearby grazing. However, our study reveals that *Fucus* may be sensitive to even non-grazing amphipods. This algal susceptibility to amphipods alone is surprising because amphipods use *Fucus* also as a habitat. Thus, *Fucus*-specimens should permanently receive the inducing cue, which should lead to a permanent induction, i.e. a de facto constitutive defence. According to Karban et al. (1999), this quasi-constitutive response should minimise for the algae the selective advantage of using water-borne cues from non-grazing herbivores to induce anti-herbivory defences. Dissipation and dilution of cues emitted by the amphipods were possible much lower in our experimental units than in the natural environment. Possibly, this leads to

cue concentrations, which in the field, would signal particularly high amphipod densities. Alternatively, *Fucus* may benefit from the ability to initiate defences as soon as the first signals of a strong grazer density are perceived, if amphipod abundances fluctuate sharply, resulting in sudden changes of grazing pressure. Testing this hypothesis requires identification of signal thresholds as well as seasonal monitoring of amphipod abundance. Finally, widespread induction response by *Fucus* should increase the number of distasteful conspecifics, which in turn may increase amphipod movements among algae, increasing amphipod visibility to their predators (Borell et al. 2004).

In conclusion, the effectiveness of lipophilic compounds as well as speed and strength of inducible anti-herbivory defences were variable in time. This variation adds complexity to algae-mesoherbivore interactions, because inducible algae remain at least to a certain extend part of herbivore's diet rather than constitutively defended algae. Therefore, inducible defence could possibly advance stability in benthic systems similar to what is known from planktonic systems (Verschoor et al. 2004).

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