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**RESEARCH ARTICLE**

# Quantitative assessment of animal-induced leaf damage: a test with three brackish crustaceans feeding on leaf detritus.

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## Abstract

1 - A computerized image analysis technique was implemented to quantify small-scale leaf damage due to different feeding strategies of macrobenthic invertebrates. Additionally, a foraging strategy index (FSI) was developed to provide a quantitative estimation of inter-specific changes in feeding strategies.

2 - Laboratory feeding trials were run with the three brackish crustaceans *Idotea baltica* and *Lekanesphaera hookeri* (Isopoda) and *Gammarus aequicauda* (Amphipoda); the effect of their feeding activities were tested on decaying leaves of *Cymodocea nodosa* and *Phragmites australis*.

3 - Opposite foraging strategies characterised the isopods: *Idotea baltica* shredded the leaf blade heavily, whereas *Lekanesphaera hookeri* scraped the leaf surface removing the waxy cuticle and associated heterotrophic microflora. Qualitative observations corresponded to consistent variations in FSI values, with *I. Baltica* showing the lowest and S. hookeri the highest FSI values, respectively. Leaves consumed by *Gammarus aequicauda*, on the other hand, showed both shredding and scraping damages, with FSI values intermediate between those observed for *I. Baltica* and *L. hookeri,* respectively.

4 - The aforementioned differences in trophic strategy were independent from the trophic resource exploited *inter-specific*.

5 - The methodology and the index herein presented were effective in providing a quantitative assessment of species-specific differences in the feeding strategy of three ubiquitous components of Mediterranean macrobenthic communities, for which only qualitative, phenomenological descriptions of feeding effects on trophic resources have been to date provided.

**Keywords:** foraging strategy, leaf detritus, intra-specific interference

## Introduction

In estuaries, lagoons, and other brackish habitats, macroinvertebrate consumers play a key role in controlling energy fluxes from primary producers. This control is exerted both directly, through grazing on living macrophytes, and indirectly, through the consumption of leaf detritus. As a matter of facts, a considerable fraction of the carbon fixed by vascular macrophytes enters a detritus pathway, influencing energy transfers along the local food chain or towards nearby marine waters (*e.g.*, Mann, 1988; Duarte and Cebrian, 1996). The microflora plays a considerable role in detritus decay, degrading refractory polymers and concentrating dissolved nutrients from the water column (Morrison and White, 1980; Moran and Hodson, 1989a, 1989b; Mancinelli *et al*, 2009 and literature cited). Yet, the accelerated fragmentation of dead vascular macrophytes induced by detritivores feeding activity is pivotal to the control of plant detritus decomposition rates, since both leaching and microbial degradation increase with decreasing particle size of leaf detritus (Wotton, 1994; Wotton and Malmqvist 2001). Invertebrate detritivores are actually made up by a diverse and heterogeneous group of taxa, ranging from polychaetes to gastropods to crustaceans. Yet, the pivotal role played by macrophagous isopods and amphipods has been repeatedly emphasized in several coastal and brackish environments (Harrison 1977; Robertson and Mann, 1980; Newell 1993, 1996). In Mediterranean coastal and brackish waters, three genera - *i.e.*, *Idotea*, *Lekanesphaera* (or *Sphaeroma*), and *Gammarus* - are an almost ubiquitous component of this group. They include species known to exploit detritus as a trophic resource as well as living macroalgae and macrophytes (Kamermans *et al*, 2002; Lapointe *et al*, 2006). The modality by which detritivores interact with their resource as been scrutinized in freshwaters. Efforts have been made to distinguish among different trophic strategies, culminating in a widely accepted classification into tropho-functional groups (‘shredders’ and ‘scrapers’, *sensu* Petersen and Cummins, 1974; Wetzel, 1975). In contrast, to date scarce efforts have been made to make a specific tropho-functional distinction for brackish and marine detritivores. Only broad, qualitative distinctions have been made between vertebrate and invertebrate consumers of senescing leaf blades (Figure 1).

In addition, non univocal terminology has been adopted in the literature, with the term ‘scraping’, for example, corresponding to different definitions such as ‘grooming’ (Zimmerman *et al*, 1979) or ‘browsing’ (Roberson and Mann, 1980). Rossi and Costantini, (2000) and Casagranda *et al*, (2006) represent noticeable exceptions, as they extented the freshwater classification for brackish environments.

The present study implements a methodology based on computerized image analysis to provide a quantitative assessment of the strategy adopted by brackish and coastal

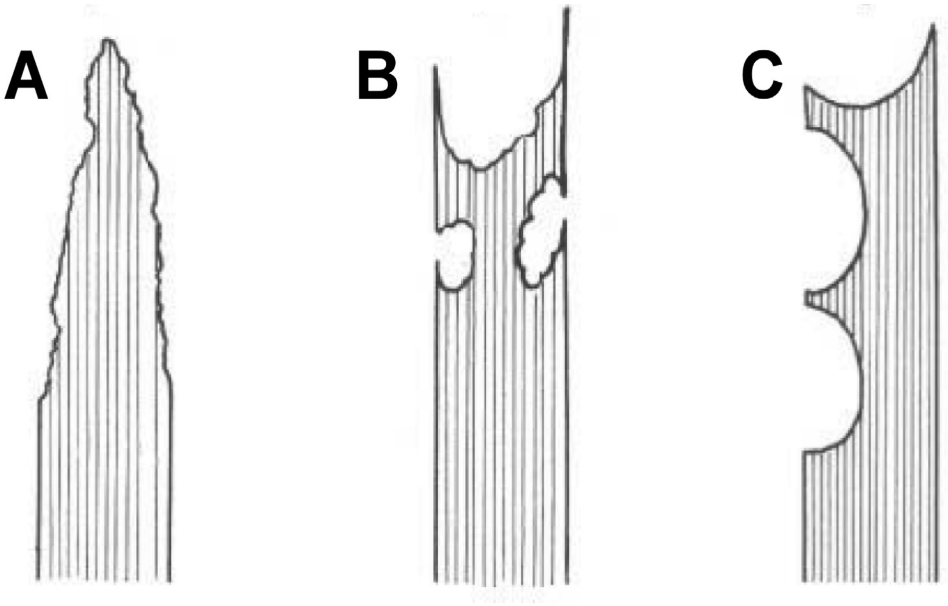

Figure 1. Damage determined by the feeding activity of three herbivores: A = *Paracentrotus lividus*; B = *Idotea hectica*; C = *Sarpa salpa* (modified from Boudouresque and Meinesz, 1982).

macrophagous invertebrates to exploit decaying leaf detritus. To this end, the methodology was tested using three species of crustacean consumers represented by the isopods *Idotea baltica*, *Lekanesphaera hookeri* and *Gammarus aequicauda*. The generality of the results was further assessed by submitting to the three species two different detrital substrates, *Cymodocea nodosa* and *Phragmites australis*.

## Material and methods

Biological materials were collected in the Acquatina Lagoon (40°26'34"N; 18°14'13"E) located in Puglia (SE Italy) on the Adriatic Sea. Complete information on the system morphology and oceanography is provided in Costantini *et al*. (2008). In brief, the lagoon is a shallow (1.2 m average depth) coastal basin with a surface area of ~0.45 km$^2$. Dense stands of the seagrass *Cymodocea nodosa* (Ucria) Ascherson cover more than 50% of the bottoms, while other seagrasses of the genera *Ruppia* and *Zostera* together with macroalgae of the genera *Ulva*, *Ceramium*, *Chondria* and *Caulerpa* are locally abundant (Maci, personal communication). *C. nodosa* represents the main source of autochthonous plant detritus to the lagoon benthic system; secondary allochthonous inputs are represented by *Phragmites australis* (Cav.) Trin. ex Steudel and by *Posidonia oceanica* (L.) Delile leaf detritus, the latter entering the lagoon during storm events.

The detritivore crustaceans *Idotea baltica* Pallas (Isopoda, Valvifera), *Lekanesphaera hookeri* Leach (Isopoda, Flabellifera), and *Gammarus aequicauda* Martynov (Amphipoda, Gammaridea) are commonly found in natural leaf detritus accumulations and in general dominate the epifaunal assemblage associated to seagrass and macroalgal beds. The amphipods *Microdeutopus gryllotalpa* Costa and *Corophium* sp., the decapod *Palaemonetes* sp., and the sphaeromatid isopod *Cymodoce truncata* Leach are other epifaunal taxa occurring in lagoon locally.

In late Fall 2005 Decaying *Cymodocea nodosa* Ucria Ascher. and P*hragmites australis* (Cav.) Trin. ex Steud. leaves were collected from clumps floating at the water surface or cumulated along the lagoon shores.

In the laboratory, leaf blades were defaunated and subsequently sonicated for 5 min in 200 ml of filtered lagoon water (Sartorius 0.45 µm; FLW hereafter) to dislodge bryozoans and other epibionts. Leaves were eventually cut into fragments long 160.0 and 80.0 mm for *C. nodosa* and *P. australis*, respectively. The different length were chosen in order to expose to invertebrate consumption a similar surface (approx. 800 mm$^2$). In addition, to minimise among-fragment variations in thickness and toughness, only the central section of the leaf blade was used, whereas both the section near the point of insertion of the blade-sheath and the blade apex were discarded. After cutting, fragments were carefully inspected under a binocular microscope, and discarded when grazing damage was detected; they were consequently oven dried (72 h, 60 °C), weighted (to the nearest 0.1 mg) and tagged individually at one end with a numbered plastic paper clip. Fragments were incubated for 7 days at 20 °C in an aerated suspension of fresh surface sediment (5 g L$^{-1}$ in FLW) to reduce inter-fragment differences in leaf surface microbial conditioning.

One week prior to the start of the experiment, *G. aequicauda*, *I. baltica* and *L. hookeri* individuals were collected at a sampling site located in the northern area of the Acquatina lagoon. In the laboratory, crustaceans were stocked in aerated 10 L. mono-specific aquaria containing FLW, and maintained on a mixed diet of *Cymodocea* and *Phragmites* leaf detritus for six days. During this period, mortality was negligible, and a macroscopic production of faecal pellets occurred for the three species.

One day before the start of the experiment, individuals were transferred in aerated plastic bowls containing 100 ml of FLW where they were starved for 24 h.

*Experimental setting*

At the end of the conditioning phase, leaf fragments were inspected and discarded when damaged areas - where the epidermis had been lost - were revealed. Subsequently, images relative to both sides of each leaf fragments were acquired using a video image analysis procedure, and the initial total area of fragments determined after image calibration (see next section).

At the start of the experiment, individuals were placed in aerated jars filled with 300 ml of FLW, and presented with a leaf fragment. For each species, a density of six individuals per jar was provided. Animals were chosen randomly and no attempts were made to discriminate between sexes; yet, great care was put in selecting wealthy adults as close as possible in size; moulting individuals were also discarded.

5 replicated jars were provided for each leaf type; five additional defaunated controls per leaf type were used to estimate changes in leaf fragments caused by factors other than consumption.

The experiment was run under natural light conditions in a controlled temperature room (20°C). Individuals were allowed to feed for 24 h. During the experiment, jars were inspected periodically; dead individuals were removed and substituted with living, starved individuals similar in size; animal substitution was stopped three hours before the end of the experiment.

After the end of the experiment, jars were inspected visually and individuals occurring on each fragment were enumerated. Fragment were collected, subjected to image analysis procedures (see next section), and dry weighted (72 h, 60°C). Subsequently, the ash-free dry mass was determined as dry mass loss after ignition (450°C, 6 h). All individuals in jars were collected, enumerated, and dry weighted.

*Image analysis*

*Acquisition and pre-processing* - Leaf fragment images were acquired by placing the fragment between two plastic slides, and scanning both sides against a white background with a flatbed color scanner (HP Scanjet 3500c) using HP Imaging and Printing ver.1.1 software. The scanner was set to generate digital RGB Truecolor (16.8 millions colors) images in .bmp format at a resolution of 600 dpi. After acquisition, images were converted to grey scale (8 bits); no post-scanning editing (*e.g.*, picture brightness, contrast and sharpness) was performed. Digital images were analyzed using the public domain software ‘ImageJ’ v.1.38 (NIH, Maryland, USA; http://rsb.info.nih.gov/ij/). Each pixel within leaf fragment images was characterized by its grey level, *i.e.*, a number ranging from 0 (black), to 255 (white). Scanned images were pre-processed by removing pixels belonging to the white background. To this end, a reference image of the empty background was acquired, and a range of grey values comprised between 192 and 255 was determined. An upper threshold was consequently set at 192 units of the grey scale, and all grey scale values ≥ 192 were replaced with the value 255.

*Calibration and measurements* - At the end of the conditioning phase, the epidermal cells were removed from the end of an intact *Cymodocea* fragment with a dissecting scalpel under a microscope. The fragment image was subsequently acquired and pre-processed according to the aforementioned procedures. Qualitative identification of the damaged area was greatly facilitated by the brownish epidermis characterising highly conditioned *Cymodocea and Phragmites* leaf detritus, whereas the underlying mesophyll tissue appears significantly paler (Figure 2). To define a standard, pre-defined threshold for grey hues of pixels characterizing damaged areas, a 100 pixels-long section was randomly drawn across the boundary between the damaged and intact area. Grey level values characterising each pixel along the section were acquired (Figures 1-2) and subjected to an unsupervised expectation-maximization classification procedure (CEM

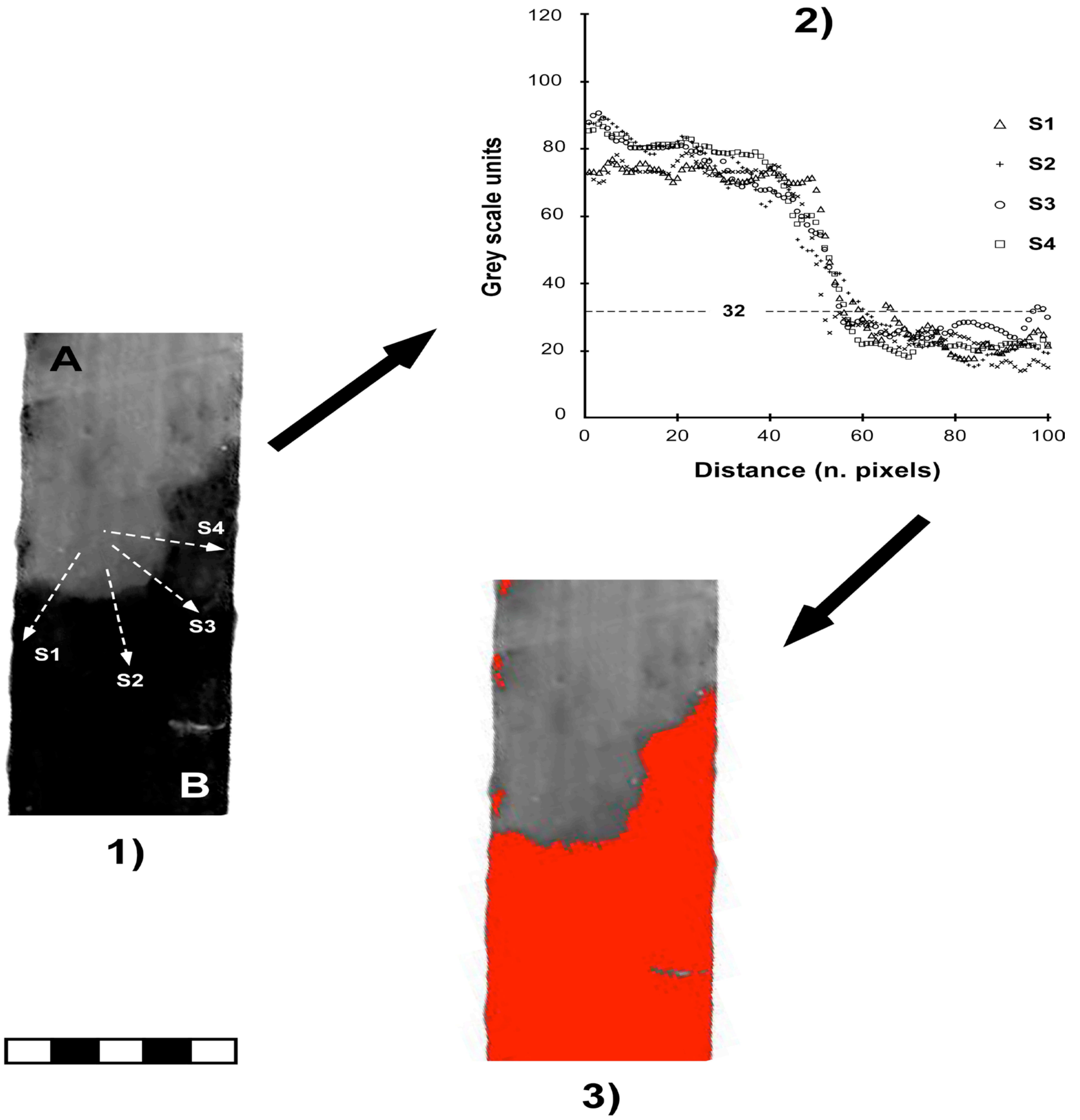

Figure 2. Synthesis of the procedure adopted to quantify the area of leaf pages damaged by the removal of the leaf epidermis and superficial tissues. 1) the epidermis of a *Cymodocea nodosa* leaf is removed by a scalpel; on a 8 bit-256 grey level image ten 100 pixel-long profiles across the intact/damaged border are drawn in Imagej, and the grey levels estimated; 2) the threshold intact/damaged area is determined by a unsupervised expectation-maximization classification procedure; 3) the original image of the leaf page is thresholded accordingly, and the damaged area is estimated. Scale bar = 5 mm. An identical procedure was implemented for *Phragmites australis* leaves.

algorithm; Celeux and Govaert 1992) into two different clusters using the public domain software ‘KlustaWin’ v.4.0 (courtesy of W. J. Heitler, University of St Andrews, UK and K. Harris, Rutgers University, USA). The intact/damaged threshold (DT in the following text) was determined as the grey level separating the two clusters. The procedure was repeated ten times. Without exception, the grey level value = 32 was determined as the DT value separating the intact (< 32) from the damaged area of the leaf blade (≥ 32), and adopted for further analyses. An identical procedure was followed for *P. australis* leaves, for which the threshold grey value was 65, respectively. After calibration, acquisition of images relative to both sides of leaf fragments was completed. Subsequently, for each fragment were determined I) the initial total area ($TA_I$), as the number of pixels characterised by grey levels comprised between 0 and 192 (excluded) and II) the initial intact area ($IA_I$), as the number of pixels characterised by grey levels comprised between 0 and 32, and 0 and 65 (excluded) for *C. nodosa* or *P. australis*, respectively. The initial damaged area ($DA_I$) was estimated as the difference between $TA_I$ and $IA_I$. $DA_I$ was calculated for both sides of the leaf.

Image acquisition was repeated at the end of the laboratory experiment, and for each fragment the final total area ($TA_F$) was estimated. The difference $TA_I$ - $TA_F$ was used to calculate the area of the leaf damaged adopting a shredding consumption strategy ($A_C$) while the area comprised between the grey values 192 and 32 (*C. nodosa*) or 65 (*P. australis*) was used to assess the damage adopting a consumption strategy limited to the removal of the epidermis and other superficial tissues of the leaf page ($DA_F$). Measurements were repeated for leaf page sides. On both occasions, measures expressed as number of pixels were transformed in square millimetres. A trophic strategy index - *i.e.*, a metrics expressing the preference of the animal towards damaging the leaf by removing whole fragments or, in contrast, by scraping the leaf page surface - was determined as

$$TSI = \frac{A_C + (DA_F + DA_I)}{A_C}$$

The index equals the unity when the animal trophic strategy is completely focused on fragmenting the leaf; the value of TSI increases as the contribution of scraping to leaf damage becomes more important.

## Results and Discussion

### *General observations*

In spite of the short duration of the experiment, both *Gammarus aequicauda* and *Idotea baltica* were characterised by a high mortality: out of a total of 60 individuals originally used for each species, 7 isopods and 11 amphipods were lost. No dead individuals were recovered; in particular, large *G. aequicauda* specimens were repeatedly observed while feeding on smaller conspecifics. In contrast, only 2 sphaeromatids were lost during the trial, and they were all recovered.

Animal abundances used in the trials corresponded to a density of 0.01 - 0.02 individuals mg$^{-1}$ dry mass (Mancinelli, unpublished data) depending on the detrital substrate. Densities were chosen as to cover the range observed for *G. aequicauda* and *I. baltica* in the field (Costantini and Rossi, 1995; Maci, unpublished data). The cannibalistic phenomenon observed for both species indicate that, under artificial conditions deprived of refugees, predation on smaller conspecifics emerges clearly (e.g., see also, among others, Andersson *et al*, 2009), indicating that cannibalistic behaviors may play an important role in determining population densities in the field (Dick, 1996; Devin and Beisel, 2007; Grigorovich *et al*, 2008).

During the experiment, gammarideans demonstrated to be the most mobile and active, free-swimming in the jar and moving along *Cymodocea* and *Phragmites* fragments. Similarly, *Idotea baltica* showed a certain degree of vagility, but tended to remain attached to fragments and only episodically to swim and crawl on the jar bottom. For both species, increased mobility was observed as an escape response to inter-individual interactions (e.g. while feeding closely on the same algal fragment).

In contrast, *Lekanesphaera hookeri* resulted the most sedentary, as specimens tended to attach to leaf fragments and gather into groups.

Qualitative observations on species vagility were confirmed by data on the fraction of specimens found associated to leaf fragments at the end of the experiment: on average, 89.8% ± 3.3 and 94.7% ± 7.3 of *L. hookeri* individuals were found on *Cymodocea* and *Phragmites* fragments, respectively, whereas 46.3% ± 5.5 and 38.3% ± 7.2 were observed for *G. aequicauda* and 63.1% ± 6.2 and 57.2 ± 6.1 for *I. baltica*.

*Trophic strategies*

*G. aequicauda* heavily grazed on *Cymodocea* and *Phragmites* fragments, shredding the leaf blade and scraping off the epidermis (Figure 3, Figure 4). Leaf fragments showed clear biting marks on the leaf edge and scraping scars were ubiquitous all over

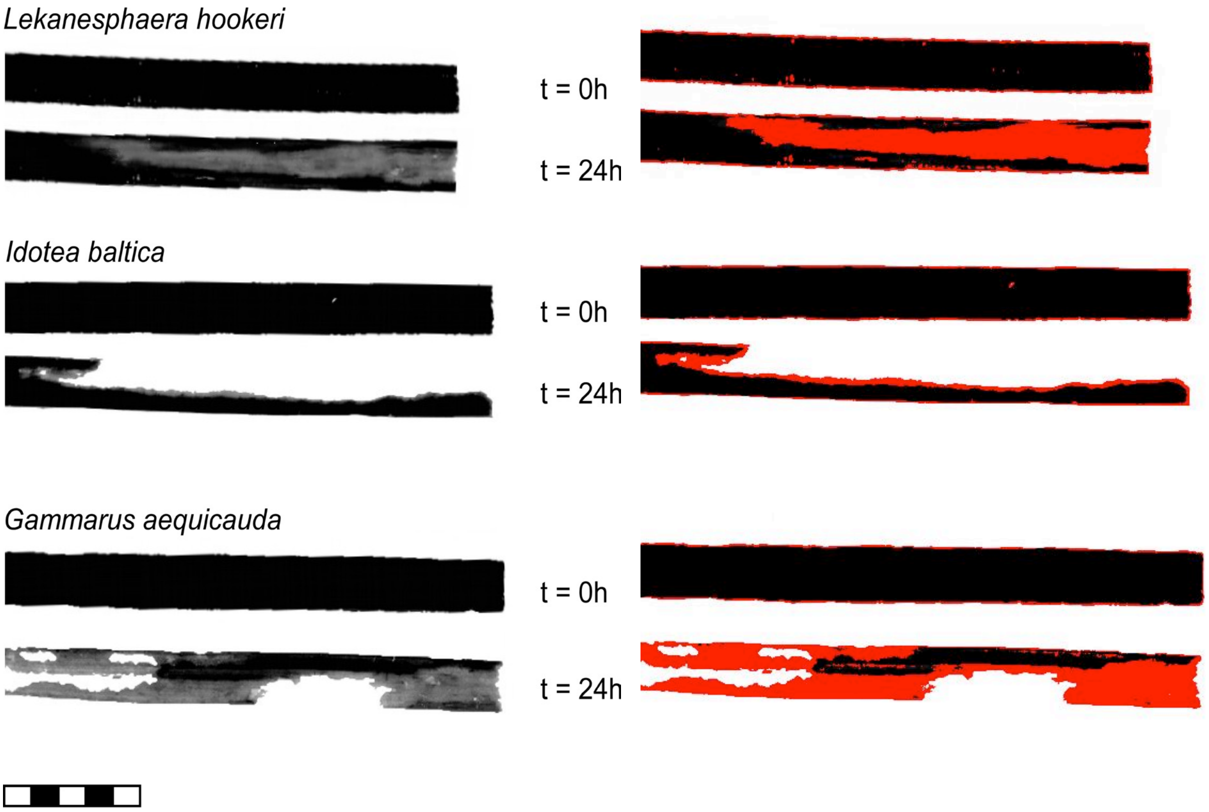

Figure 3. Damage determined by *Lekanesphaera hookeri*, *Idotea baltica* and *Gammarus aequicauda* on a *Cymodocea nodosa* leaf after 24 hours. Left = original images; Right = thresholded images. The area where the epidermis has been removed is shown in red. Scale bar = 10 mm.

the leaf surface. Similarly, Idotea baltica produced heavy shredding damages on the leaf page independently from the resource; in contrast to the amphipod, however, scraping was reduced and limited to the edge of the shredded areas. *Lekanesphaera hookeri* trophic activity resulted the least destructive: biting marks and shredding of the leaf blade resulted far less evident, whereas the surface resulted clearly scraped. These phenomenological considerations were quantitatively expressed by variations in the TSI index (Figure 5). Independently from the detrital substrate, *Idotea baltica* showed the lowest TSIs, while *Lekanesphaera hookeri* was characterized by the highest. According to the freshwater tropho-functional classification (Cummins, 1974). *Idotea* can be considered as a true shredder, impacting heavily the leaf by fragmenting it while exerting a negligible effect on the surrounding leaf page; conversely, *Lekanesphaera* can be classified as a scraper, grazing the leaf page surface, removing the soft, decomposed epidermis together with the autotrophic and heterotrophic microflora colonizing it. Such a distinction in the trophic strategy adopted in interacting with the detrital substrate, implies, at a broader functional scale, a distinct typology of interaction when the two species acts as mesograzers, feeding on living plants: *Idotea* may feed heavily on macrophytes, while leaving the surrounding microepiphyton relatively undisturbed, whereas *Lekanesphaera* my establish a stronger interaction with the latter. A number

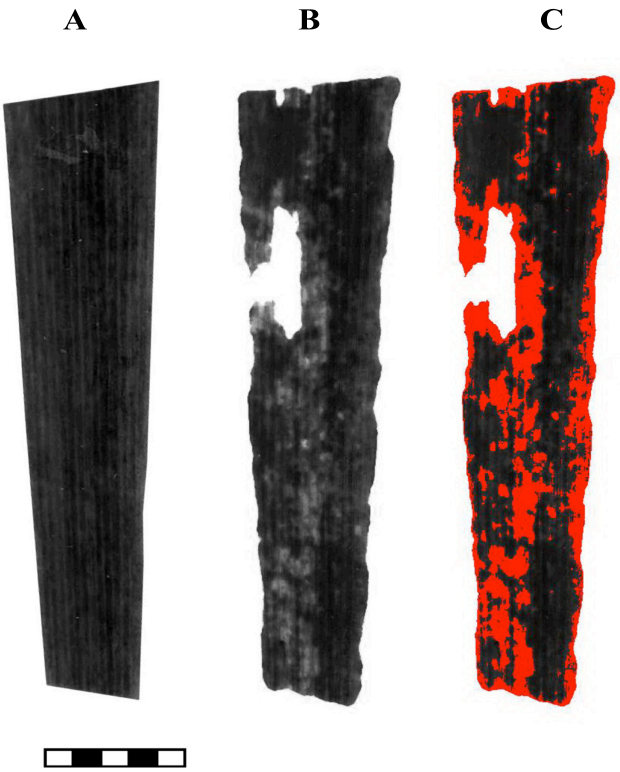

Figure 4. Damage determined by *Gammarus aequicauda* on a *Phragmites australis* leaf after 24 hours. The area where the epidermis has been removed is shown in red. A = leaf fragment at t = 0; B fragment at t = 24 hours; C = thresholded image. Scale bar = 25 mm. Damage patterns produced by *Idotea baltica* and *Lekanesphaera hookeri* trophic activity were similar to those observed on *Cymodocea nodosa* and are not shown.

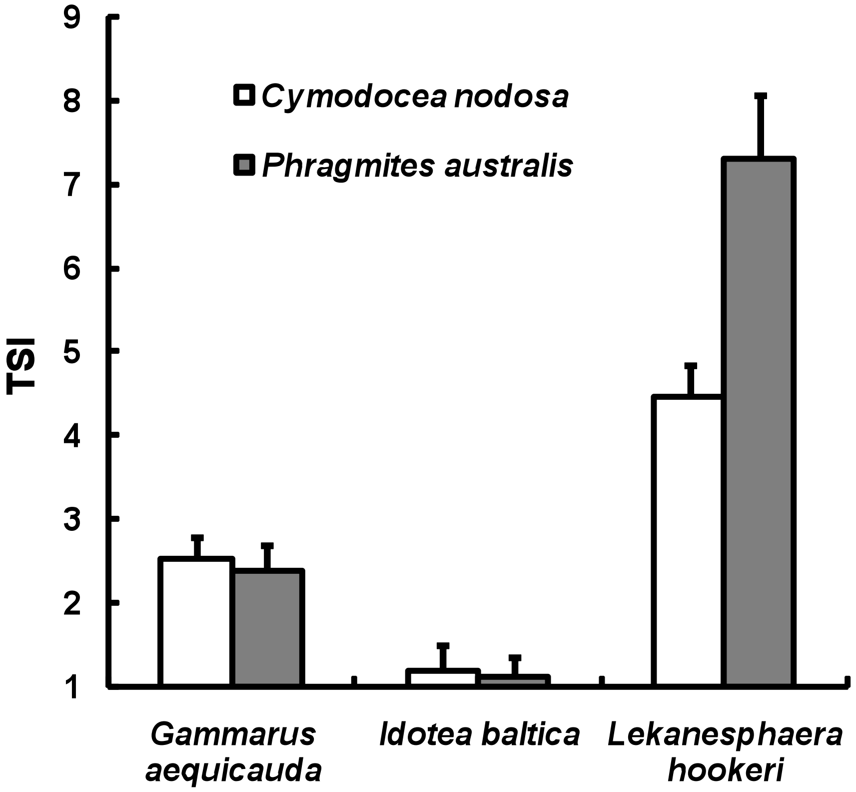

Figure 5. Trophic strategy index (TSI) calculated for *Gammarus aequicauda*, *Idotea baltica*, and Lekanesphaera hookeri feeding on *Cymodocea nodosa* and *Phragmites australis* leaf detritus. For each detritus type, TSI values differ significantly among consumer species (Tukey HSD post-hoc test after 2-way ANOVA with Factor 1 = detritus type and Factor 2 = consumer species: P always < 0.05). Bar = 1 SE

of studies confirm such a strategy for *Idotea* (Bostrom and Mattila, 2005 and literature cited) whereas there are some noticeable exceptions (Jaschinski and Sommer 2008 and literature cited). For *Lekanesphaera* information are scant, yet its preference for epibionts has been clearly acknowledged (Kamermans *et al.* 2002). *Gammarus aequicauda* showed intermediate FMI values. Extending what already introduced for isopods, *Gammarus* can be considered both a shredder and a scraper. Thus, it has the potential to affect both the detrital substrate and the microbial component associated to its surface; from a mesograzer point of view, *Gammarus* can affect both macrophytes and epibionts and microepiphytes, confirming what observed by Kamermans *et al*, (2002). The present results confirm the high trophic plasticity of *Gammarus* spp. and their potential to exert a wide spectrum of functional roles within detritus-based and algal based food webs.

## Conclusions

In conclusion, the methodological procedure and the index herein presented were effective in providing a quantitative assessment of species-specific differences in the feeding strategy of three ubiquitous components of Mediterranean macrobenthic communities, for which only qualitative, phenomenological descriptions of the respective effects on trophic resources have been to date provided. Further studies are needed to assess whether biotic interactions - e.g., risk of predation, intra - or inter-specific competition - or abiotic constraints - e.g., temperature – can influence FMI values, ultimately varying the trophic strategy of the animal.

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