



# Past and present grazing boosts the photo-autotrophic biomass of biofilms

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**ABSTRACT:** Little is known about the long-term consequences of grazing effects on microphytes. This study tested density-dependent responses to grazer removal on the biomass (chlorophyll *a*, chl *a*) and composition of natural high rocky-shore biofilms over a 7 mo period. Gastropod snails *Melarhaphé neritoides* graze entirely within circular halos generated in biofilms surrounding their refuges. The experiment crossed 3 levels of original snail density per halo with 3 levels of grazing intensity (generated by 100, 50 and 0% snail removal). Areas inside halos from which all snails had been removed sustained significantly higher chl *a* than never-grazed control areas outside the halos. This effect of grazing history was still present after 7 mo, suggesting that past grazing had an enduring positive influence on biofilm biomass. Against expectation, chl *a*-biomass was not increased by removing snails, regardless of original grazer density. Half- and fully-grazed halos peaked to a higher chl *a* than ungrazed halos in spring. Grazing did not affect the presence of major biofilm taxonomic groups, although it did alter their relative contributions. Never-grazed areas were covered by a thick biofilm-detritus complex and had proportionally more filamentous cyanobacteria than grazed areas, which sustained abundant clusters of coccoid cyanobacteria and lichen within micro-pits inaccessible to snail radulae. The present study shows that effects of grazing history are not exclusive to macrophytic systems. Grazers boosted the concentration of micro-autotrophs relative to non-chl *a* biofilm constituents, probably by removing an unproductive biofilm canopy and facilitating light and nutrient penetration for new growth.

**KEY WORDS:** Standing stock · Epilithic biofilm · Micro-algae · Grazing · Refuge · Rocky shore · Detritus · Littorina

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## INTRODUCTION

Grazing history is known to affect terrestrial plant growth, with higher vegetative biomass or seed abundance in areas that were previously grazed, compared to areas with minimal grazing history (Paige 1999, McIntire & Hik 2005). Higher biomass may arise from grazing having altered the species assembly or the ecotypes of vegetation communities, or through it stimulating plant overcompensation (Noy-Meir et al. 1989, Paige 1999, Agrawal 2000, McIntire & Hik 2005). The effect of grazing history on vascular plant systems may take years to

emerge after grazer exclusion (McIntire & Hik 2005), after which it can persist for decades or even become irreversible (Noy-Meir et al. 1989, Painter et al. 1993). Studies of grazing history have focused predominantly on vertebrate grazers and terrestrial macrophytic systems. There is some evidence, however, that a history of grazing can also affect the composition of microphytic aquatic communities. Peterson & Boulton (1999) found that freshwater biofilms with a history of tadpole grazing differed in physiognomy and species composition from those without. Grazing history has not been examined for effects on naturally growing biofilms.

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The effects of grazing on vegetation community composition generally occur at high grazer abundance (Noy-Meir et al. 1989, Huntly 1991, Sommer 1999, Hillebrand 2008). A history of heavy grazing might therefore have a stronger effect than a history of light grazing. Most field exclusions of grazers have been applied to densely grazed areas, ignoring possible interactions with density (McIntire & Hik 2005). Experimental exclusions of molluscan grazers from rocky shores typically result in profuse growth of microalgal biofilms, which is frequently coupled to a change in biofilm community composition (e.g. Castenholz 1961, Nicotri 1977, Thompson et al. 2004) and subsequent macro-algal colonisation (Jenkins et al. 2001, Coleman et al. 2006). A number of studies have tested for effects of intermediate grazing intensities on biofilm primary succession, using artificial settling surfaces or highly moderated substrata (Castenholz 1961, Nicotri 1977, Hunter & Russell-Hunter 1983, Murphy & Underwood 2006). Three studies have examined the response of natural biofilms to intermediate grazing intensity (Branch & Branch 1981, Thompson et al. 2000, 2004), although without testing for effect of grazer removal across sites of differing original densities.

Long-term effects of grazing are likely to follow from persistent grazing pressure, for example where grazers preferentially feed close to preferred refuges. Visible 'grazing halos' may then form around the refuge, clearly delineating the foraging range. Examples of grazing halos include algae-feeding reef fish and sea urchins (Ogden et al. 1973, Macia & Robinson 2005), and micro- and macroalgae-feeding snails of intertidal rocky shores (Stafford & Davies 2005, Underwood & Murphy 2008). Grazing halos offer opportunities for measuring grazer impacts on plant communities while minimising the influence of spatial and environmental variation on the crop community. Effects can be measured along a continuous gradient from intensely

grazed, close to the refuge, to never grazed, outside the halo (e.g. McIntire & Hik 2005, Stafford & Davies 2005, Underwood & Murphy 2008). Studies examining halo-linked biofilm grazing on rocky shores report negative effects on biofilm biomass inside halos, and some document an increasing change in community composition with proximity to the grazer refuge (Williams et al. 2000, Stafford & Davies 2005, Underwood & Murphy 2008).

In the present study, we report biofilm responses to manipulations of grazing pressure by the littorinid snail *Melarhaphé neritoides* (L.) which colonises the upper reaches of rocky shores. During tidal emersion and when the substratum is dry, *M. neritoides* take refuge in cracks and crevices to prevent desiccation, dislodgement and predation (Myers & McGrath 1993, Cronin et al. 2000, Stafford & Davies 2005). Foraging is centred on the refuge, resulting in high grazing pressure in the immediate vicinity, and a visibly lighter coloured grazing halo surrounded by a darker never-grazed biofilm (Fig. 1a,b). Larger refuges support more occupants and consequently have larger halos than small refuges. Grazing is confined to the area inside the halo. *M. neritoides* snails rarely move outside the halo, although they are physically capable of travelling  $20 \text{ cm h}^{-1}$  during migration (Stafford & Davies 2005).

Micro-algae and bacteria that colonise hard substrata are embedded in a biofilm formed by secretion of gel-like extracellular polymeric substances (EPS; Decho 2000). Biofilms of rocky shores contain bacteria, cyanobacteria, diatoms and macro-algal germlings, as well as lichen on the high shore (Bärlocher & Murdoch 1989). The majority of biofilm cells, by mass, are photosynthetic, contributing to the primary productivity of rocky shores (Thompson et al. 2004). On the high shore and the 'splash zone' above the highest tides biofilms are often the most important food source for grazers (Thompson et al. 2000). Biofilms in temperate climates



Fig. 1. (a) Snail grazing halos, seen as light areas in the darker biofilm, at Totland Bay sea wall. A square section centrally in the image has been artificially brushed clean of biofilm. (b) Grazing halo of *Melarhaphé neritoides* (7 ind.) occupying a central refuge. (c) Normalised difference vegetation index image of the same halo; dark areas (immediately outside the halo) have less chl *a* than light areas (inside the halo)

grow fastest over the winter period of generally wet substratum and low desiccation and light stresses (Underwood 1984, Hill & Hawkins 1991). Chlorophyll *a* (chl *a*) is ubiquitous to all microphytes and the most widely used proxy for biofilm biomass (Hill & Hawkins 1991, Hawkins & Hartnoll 1983b, Mak & Williams 1999, Thompson et al. 2005, Murphy et al. 2006).

The present study tracked long-term biofilm responses to partial and full removals of *Melarhaphé neritoides* from rocky shore areas with varying grazer density. These periwinkles are the only resident grazers of upper littoral fringe in the study area, where they graze epilithic biofilms exclusively within halos around refuges. We tested for responses in community composition, coverage and chl *a* biomass of biofilms. We removed either 0, 50 or 100% of the snails from sites with low, medium and high original grazer density, and tracked the biofilm response over 7 mo. This fully-crossed design permitted testing for effects of reduced grazing and for evidence of grazing history at different levels of original grazing intensity. Tests for effects of grazing history sought differences between the chl *a*-biomass inside (grazing history) compared to outside (no grazing history) halos with full snail removal.

## MATERIALS AND METHODS

*Melarhaphé neritoides* and biofilm dominated assemblages were studied on the concrete sea wall at Totland Bay, on the west coast of the Isle of Wight, UK (50° 41' 05.29" N, 1° 32' 37.49" W). Totland has diurnal tides and a spring tidal range of ~2.5 m. Sampling took place within a homogenous 100 m length of shore, 1.5 to 2.5 m above spring high water in a narrow band within the splash-zone. This upper part of the sea wall is pitted with holes and crevices into which *M. neritoides* aggregate, with 2 to 35 ind. refuge<sup>-1</sup> and ~50% of holes and crevices occupied by snails. Snails average 4.0 mm in shell length and their densities range from 20 to 80 m<sup>-2</sup>. They recruit moderately all year round with peak months differing between years. Snails rarely change refuge. In 3 sets of 10 d observations of 120 marked individuals at study location, only 5.9% snails switched refuge. The upper wall was selected for study because it had no other periwinkles, thereby excluding inter-specific interactions, and because biofilms between halos had minimal recent grazing history (monitoring showed unmarked halos remained separated and visible for 12 mo prior to experimentation).

**Effect of grazing on biofilm community composition.** The biofilm was examined inside versus outside grazing halos for differences in major autotrophic microbial groups. High performance liquid chro-

matography (HPLC) was used for quantitative comparison of major pigment groups and scanning electron microscopy (SEM) for qualitative visualization. HPLC identified relative contributions of 'marker pigments' specific to cyanobacteria (e.g. pigments myxoxanthophyll and equinone), diatoms (diadinoxanthin), green algae (chl *b*) and brown and green macroalgae (violaxanthin). Conventionally, relative contribution of each algal group to the sample community is derived from concentration of marker pigment relative to the value for chl *a* (Wright et al. 1991). In February 2008 (mid experiment) 2 rock chips, 1 for HPLC (20 × 20 mm) and 1 for SEM (15 × 15 mm), were taken inside (grazed) and 2 chips were taken outside (never grazed) each of 5 random halos (snail density: 6 to 12 halo<sup>-1</sup>) located in close proximity to experimental halos. HPLC chips were frozen (-70°C) and SEM chips were fixed in 2.5% glutaraldehyde in 22 µm filtered seawater. SEM samples were viewed in a Jeol 5600LV scanning electron microscope with low vacuum setting. Samples were dipped in distilled water to remove glutaraldehyde before observation. HPLC analysis measured the pigment concentration per area (µg cm<sup>-2</sup>) using a Shimadzu HPLC comprised of a solvent delivery module (LC-10ADVP) with system controller (SCL-10AVP), a photodiode array (SPD-M10AVP) and a fluorescence detector (RF-10AXL). Chromatographic separation was carried out using a C18 column for reverse phase chromatography (Supelcosil, 25 cm long, 4.6 mm in diameter and 5 µm particles). The solvent gradient followed Kraay et al. (1992) with a flow rate of 0.6 ml min<sup>-1</sup> and an injection volume of 100 µl. Rock chip-pings were extracted with 95% cold buffered methanol (2% ammonium acetate) overnight at -20°C, in the dark. Identification and calibration of the HPLC peaks was confirmed with chl *a* & *b* and β-carotene standards from Sigma-Aldrich (<http://sigmaaldrich.com>), and chl *c*, fucoxanthin, diadinoxanthin, diatoxanthin, lutein, zeaxanthin and phaeophytin-*a* standards from DHI Lab products (<http://c14.dhigroup.com>). Scytonemin was identified by its retention time and its absorption spectra at 370 nm. Chlorophylls and carotenoids were identified and quantified with photodiode array. Pheophorbides and pheophytins were identified and quantified with the fluorescence detector.

**Biofilm response to snail removal and grazing history.** A series of natural grazing halos with a single central refuge was identified in early October 2007 and an initial record was taken of the number of occupants per halo. Densities ranged from 0 to 31 snails halo<sup>-1</sup>. Halos from 5 clearly-separated levels of snail density were selected for experiments; these had 1, 4, 8–11, 17–21 or 27–31 snails halo<sup>-1</sup>. Three levels of grazer 'Density' (4, 8–11, and 17–21 snails halo<sup>-1</sup>) were cross factored with 3 levels of 'Grazing intensity' ('Zero',

'Half' or 'Full' natural grazing intensity). The treatments of Zero, Half and Full grazing intensity were generated by manually removing respectively 100, 50 or 0% of snail occupants per halo. Each treatment combination had 5 replicate halos, requiring 45 halos in total. Treatments were randomly allocated to halos. Snail numbers per halo were checked and corrected fortnightly. This maintained densities on average  $5.1 \pm 3.5\%$  ( $\pm$ SE) from the target. Grazing halos were observed monthly from October 2007 to May 2008 for changes in area, encompassing the time of maximum biofilm formation (Thompson et al. 2004) and high shore ephemeral algal biomass (Hawkins & Hartnoll 1983a). Each halo was photographed with a digital camera mounted on a frame to sample  $40 \times 40$  cm areas. Images were analysed using Image-J software (<http://rsb.info.nih.gov/ij/>). The edge of the halo was consistently detectable as a distinct colour change from light to dark. The grazed area was measured against the known 40 cm width of observation frames after digitally outlining the halo edge.

Chl *a* was estimated using Colour InfraRed photography (CIR) following Murphy et al. (2006). Traditional methods for chl *a* estimation, such as removing rock chips and brushing the substratum, are destructive and impractical at sampling scales <10 mm. CIR produces non-invasive remote-sensing images that can be sub-sampled for chl *a* in any area of interest and to the scale required. The technique is equally sensitive to chl *a* within different biofilm assemblages (Murphy & Underwood 2006).

A 2.1 mega-pixel Nikon Coolpix 950 digital camera was used for image capture. The infrared filter was removed to adapt the camera for remote sensing (Chapman 2007). The camera was mounted in an aluminium frame producing  $50 \times 50$  cm images of the substratum. CIR images were collected bimonthly between November 2007 and May 2008, except for March when stormy weather conditions precluded observation. Two spatially identical photographic images were taken in quick succession, one through a red filter (red: 660 to 700 nm) where chlorophyll absorbs and the other through a near infrared filter (NIR: 750 to 950 nm) where chlorophyll and the surface reflects. Image-J software was used to convert photos into pixel values. Each pixel then yielded a Normalised Difference Vegetation Index:  $NDVI = (NIR - red)/(NIR + red)$ . NDVI is the most widely used vegetation index in remote sensing, and is directly proportional to the amount of chl *a* on a hard surface (Rouse et al. 1974). NDVI readings were finally converted into chl *a* values using a regression equation of chl *a* vs. NDVI. The regression was obtained from CIR images and subsequent chlorophyll extractions with 100% methanol (Thompson et al. 1999) for a series of Totland biofilm samples on

rock-chips (regression analysis—equation:  $\ln(\text{chl } a, \mu\text{g cm}^{-2}) = 3.46 \ln(\text{NDVI}) + 6.95$ ;  $F_{1,51} = 187.6$ ,  $p < 0.001$ ;  $r^2 = 0.79$ ). Fig. 1b,c shows a digital photograph and a NDVI image of an experimental halo. Chl *a* was sampled using such NDVI images, from 3 pooled observations of 220 mm<sup>2</sup> areas inside (evenly dispersed) and 3 outside each halo (evenly within 50 mm of halo). Our method estimates chl *a* with a precision of 7.3% (unpubl. laboratory tests by the authors).

NDVI images of 30 random halos from April 2008 (10 per grazing intensity) were used to examine whether grazing accentuated the small-scale variation in biofilm chl *a* biomass. Ten 25 mm<sup>2</sup> observation spots were sampled for chl *a* inside and outside each halo (totalling 600 observations). The coefficient of variation for each set of 10 chl *a* observations was used as a proxy for spatial variation. A 2-way fully crossed analysis of variance (ANOVA) tested for effects of position (inside vs. outside halo) and grazing intensity on the coefficient of variation.

**Statistical analysis. Effect of grazing on community composition:** Paired-sample *t*-tests compared the relative contribution of each pigment from HPLC analysis to pigment pools inside versus outside halos ( $n = 5$  halos). To standardise comparisons, the analysis was based on ratios of pigment to chl *a* ( $\mu\text{g pigment cm}^{-2}$ :  $\mu\text{g}^{-1}$  chl *a* cm<sup>-2</sup>).

**Effect of grazer density on halo area:** Repeated-measures analysis of covariance (rmANCOVA) tested for effect of observation Month on the linear relationship between  $\ln$  halo area and  $\ln$  snail density (covariate factor). The analysis was based on data for all halos with 0% removal, including 5 halos of the smallest density level of 1 snail halo<sup>-1</sup>, and 3 (the only available) halos for the largest density level of 27 to 31 snails halo<sup>-1</sup>. The overall regression slope was tested for deviation from 1.0 by a 1-tailed *t*-test using one mean of all months per halo.

**Effect of grazer removal on halo area and chl *a* biomass:** Repeated-measures analyses of variance (rmANOVAs) tested for interacting effects over time of past grazer density and present grazing intensity on halo area and chl *a* concentration. The 3 cross-factors were the original (October) number of snail occupants of the halo ('Density': 4, 8–11, 17–21), grazing intensity ('Intensity': Zero, Half and Full grazing) and 'Month' (5 for halo area and 4 for chl *a*). May was excluded from the global analyses because the areas of several of the Zero grazing-intensity halos had shrunk to 0, which induced severe heterogeneity of variance. Each individually identified halo was nested in Intensity  $\times$  Density and measured repeatedly across months. Effects of grazing intensity induced by snail removals were further explored with Helmert orthogonal contrasts. These partitioned the sums of squares of the Grazing Intensity main effect and the sums of squares of its

interaction with Month, contrasting ungrazed (Zero grazing) to grazed (pooled Half- and Full-grazing), and contrasting between grazed regimes (Half vs. Full grazing). In order to control for natural variation in chl *a* along the shore, its response to treatments was measured from the difference inside to outside each halo (chl  $a_{in}$  – chl  $a_{out}$ ). In the 17 to 21 density level, 2 halos (one each for Full and Zero grazing) had to be discarded after disruption by runoff following cliff erosion during the experiment, leaving this density level with only 13 halos for analysis. The resulting imbalance was accommodated by Type-II adjusted sum of squares (e.g. Doncaster & Davey 2007).

**Effect of grazing history.** Effect of grazing history was examined by comparing chl *a* content inside (with grazing history) to outside (without grazing history) the grazing halos with Zero grazing intensity. The 3 cross factors were grazing Intensity, Month and 'InOut' (inside or outside grazing halo). Halos were nested in InOut  $\times$  Density and repeatedly measured across months. The response variable, chl *a*, was ln-transformed to achieve homoscedasticity.

## RESULTS

### Community composition

Never grazed areas had a generally thick surface cover of filamentous cyanobacteria, EPS and abundant detrital matter (Fig. 2a,b) with occasional coccoid cyanobacteria, colonies of lichen (probably *Verrucaria maura* Wahlenberg) and filaments of green algae (*Ulothrix* sp.). Although grazed substrates were generally without biofilm on protruding surfaces (Fig. 2c,d), lichen and coccoid cyanobacteria remained in micro-pits and cracks inaccessible to the radulae of snails (Fig. 2d). Filamentous growth forms and detrital cover were generally absent inside halos.

HPLC analysis revealed 12 pigments, all found inside and outside grazing halos (Table 1). These included scytonemin (common to cyanobacteria and lichen and a known anti-UV agent: Büdel et al. 1997), lutein (red and green algae), violaxanthin (brown and green macroalgae), chl *b* (green algae) and zeaxanthin (major in cyanobacteria, minor in green algae). Five

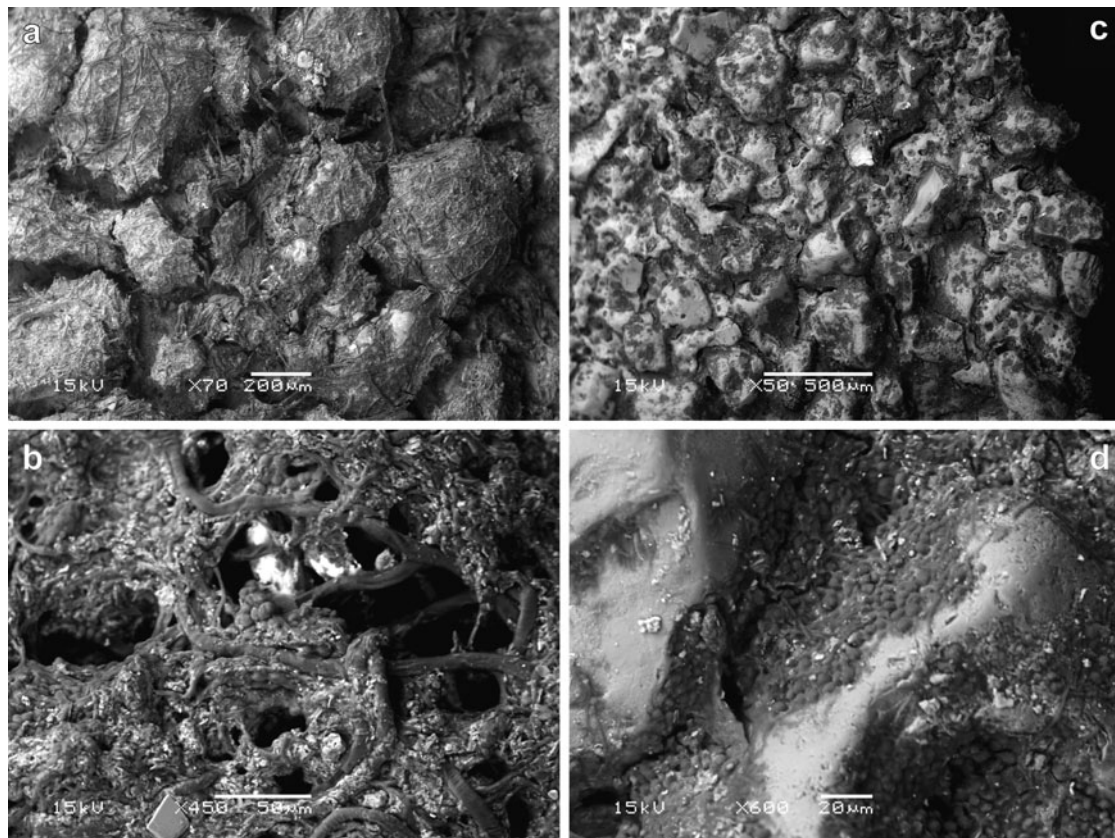


Fig. 2. Scanning electron micrographs of biofilms outside and inside grazing halos. (a,b) areas outside halos (70 and 450 $\times$  magnification, respectively) covered by a thick carpet of filamentous cyanobacteria and detrital matter. (c) Inside a halo (50 $\times$  magnification), showing the denuded conglomerate pattern of the concrete; dark areas are colonies of biofilm. (d) Inside a halo (600 $\times$  magnification), showing the protruding ends of sand grains grazed clear of biofilm, and abundant colonies of lichen and coccoid cyanobacteria left in cavities between grains

Table 1. Mean ( $\pm$ SE) pigment concentrations of biofilm samples taken inside and outside 5 *Melarihaphe neritoides* grazing halos. The analysis was done using High Performance Liquid Chromatography. Pigment concentrations are expressed relative to the sample chl *a* content (by mass,  $\mu\text{g pigment cm}^{-2}/\mu\text{g chl a cm}^{-2}$ , except scytonemin, which is by areas of peaks in HPLC chromatograms). Paired *t*-tests compare concentrations inside to outside halos. **Bold:** significant

Pigment	Inside	Outside	<i>t</i>	<i>p</i>
$\beta$ -carotene	0.048 $\pm$ 0.012	0.197 $\pm$ 0.049	3.44	<b>0.026</b>
$\beta$ - $\epsilon$ -carotene	0.001 $\pm$ 0.001	0.011 $\pm$ 0.003	3.37	<b>0.028</b>
Cantaxanthin	0.013 $\pm$ 0.005	0.137 $\pm$ 0.025	5.57	<b>0.005</b>
Chl <i>b</i>	0.051 $\pm$ 0.028	0.036 $\pm$ 0.014	0.42	0.693
Equinone	0.077 $\pm$ 0.032	0.520 $\pm$ 0.077	4.45	<b>0.011</b>
Lutein	0.038 $\pm$ 0.019	0.030 $\pm$ 0.016	0.32	0.768
Myxoxanthophyll	0.042 $\pm$ 0.013	0.534 $\pm$ 0.129	3.71	<b>0.021</b>
Neoxanthin	0.002 $\pm$ 0.002	0.006 $\pm$ 0.004	1.10	0.335
Scytonemin	1.451 $\pm$ 0.284	2.474 $\pm$ 0.441	1.52	0.203
Siphonin	0.055 $\pm$ 0.015	0.036 $\pm$ 0.001	1.28	0.270
Violaxanthin	0.001 $\pm$ 0.001	0.006 $\pm$ 0.003	1.19	0.299
Zeaxanthin	0.033 $\pm$ 0.014	0.019 $\pm$ 0.008	0.81	0.462

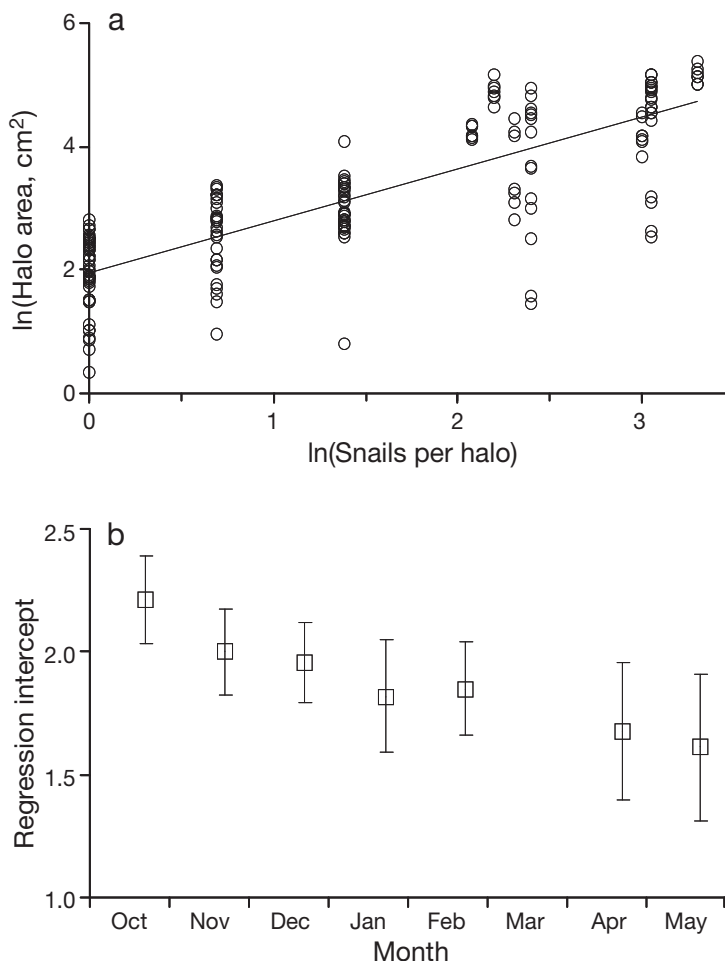


Fig. 3. (a) Ln-ln response of mean halo area to snail density for 23 experimental halos without snail removal, observed in 7 mo from October 2007 to May 2008. (b) Monthly intercept values and SE of the regression

pigments had higher content outside than inside grazing halos: myxoxanthophyll, cantaxanthin, equinone,  $\beta$ -carotene and  $\beta$ - $\epsilon$ -carotene (Table 1). All are cyanobacteria pigments, though  $\beta$ - $\epsilon$ -carotene can be found in many groups. The result suggests that cyanobacteria were proportionally more dominant outside than inside grazing halos. No other compositional differences could be detected.

### Grazing area per snail

Halo area increased with snail density per halo, for halos with no snail removal (Fig. 3a,  $r^2 = 0.66$ ,  $p < 0.001$ . Equation:  $\ln(\text{halo area, cm}^{-2}) = 0.90 \ln(\text{snails halo}^{-1}) + 1.86$ ), with a gradient of increase that was invariant between months (rmANCOVA, interaction term 'Month  $\times$  Density':  $F_{6,136} = 0.43$ ,  $p = 0.86$ ). The overall regression gradient of  $\ln$  Area against  $\ln$  Density did not differ from 1.0 (1-sample *t*-test  $t_{26} = 1.07$ ,  $p = 0.23$ ), indicating a constant halo area per snail regardless of snail density, set by the regression intercept. The value of this intercept declined steadily through the season, from 9.1 cm<sup>2</sup> in October to 5.0 cm<sup>2</sup> in May (Fig. 3b, rmANCOVA main effect of Month:  $F_{6,136} = 6.17$ ,  $p < 0.001$ ).

### Effect of snail removal on halo area

Halo areas of all treatments gradually declined from October (Fig. 4). ANOVA showed no main effect of, or interactions with, original snail density, but a clear effect of grazing intensity (levels of snail removal) that depended on month (Table 2). Orthogonal contrasts showed an overall and a month-dependent difference between the ungrazed and grazed regimes. The halos with Zero grazing intensity shrank substantially faster than those with Half grazing intensity, particularly from February to May 2008 (Fig. 4). Halos with Half grazing intensity had a reduction in area that only slightly, although cumulatively, exceeded the reduction in area of halos with Full grazing intensity, and was most marked in April to May 2008. Halos with Zero grazing were almost completely overgrown by the end of the trial, with an average 96% reduction in area after 7 mo, compared to 47% for halos with Half grazing and 24% for halos with Full grazing. Halos all expanded in February 2008 (Fig. 4), during an unseasonably warm spell when biofilms desiccated and began peeling from halo edges.

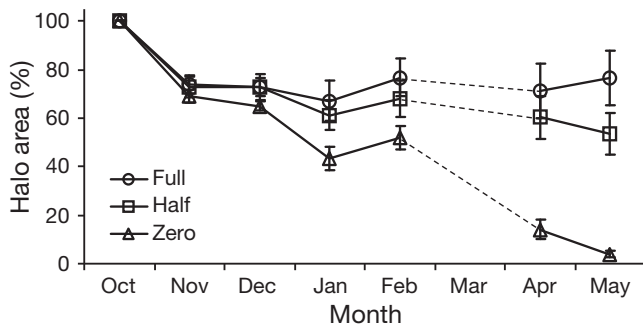


Fig. 4. Mean  $\pm$  SE change in area of grazing halos, as a percentage of original halo area, between October 2007 and May 2008, for halos with Full, Half and Zero grazing intensity (generated by removing respectively 100, 50 and 0% of original number of snails per halo)

### Effect of snail removal on chl *a* biomass

The timing of chl *a*-biomass maxima depended on grazing. Grazed surfaces (inside Full- and Half-grazing halos) increased continually to a chl *a* peak in April 2008, while ungrazed and never-grazed surfaces (inside Zero grazing-intensity halos and outside halos of all treatments) had an earlier peak in January 2008 (Fig. 5). Grazed areas reached higher peaks in chl *a* (mean  $\pm$  95% CI  $\mu\text{g chl } a \text{ cm}^{-2}$  for April: Full grazing inside:  $3.7 \pm 0.81$ ; Half grazing inside:  $3.6 \pm 0.85$ ) than never-grazed areas (Fig. 5a,b, January: Zero grazing outside =  $2.5 \pm 0.95$ ; Half grazing outside =  $2.7 \pm 0.92$ ; Full grazing outside:  $3.3 \pm 0.97$ ). ANOVA on the difference in chl *a* inside to outside the halo showed an effect of grazing intensity that depended on month, and orthogonal contrasts identified a month-dependent

difference between ungrazed and grazed regimes (Table 3). Further contrasts between months revealed that the ungrazed–grazed contrast differed between April and the other months (Fig. 5a–c, contrast  $\times$  contrast interaction:  $F_{1,102} = 17.35$ ,  $p < 0.001$ ). For Full and Half-grazing, the difference in chl *a* inside to outside was substantially higher in April than in other months, in contrast to Zero-grazing where it varied little between months. Chl *a* in April was twice as high in grazed (mean chl *a* inside Full- and Half-grazing halos =  $3.64 \mu\text{g chl } a \text{ cm}^{-2}$ ) than never-grazed (mean chl *a* outside Full- and Half-grazing halos =  $1.82 \mu\text{g chl } a \text{ cm}^{-2}$ ) areas. A sample of 30 halos in April showed chl *a* distribution was more variable inside than outside halos, with no effects of grazing intensity (2-way ANOVA on chl *a* coefficient of variation. Factor 'In vs. Out halo':  $F_{1,54} = 6.14$ ;  $p = 0.016$ . Factor 'Grazing Intensity':  $F_{1,54} = 2.4$ ;  $p = 0.1$ ). The coefficient of variation of chl *a* averaged 6% higher inside ( $17.5 \pm 3.1\%$ ) than outside halos ( $11.6 \pm 3.6\%$ ).

### Effect of grazing history on chl *a* biomass

Effect of grazing history is illustrated in Fig. 5c. rmANOVA on the ln-transformed chl *a* content of halos with Zero grazing intensity showed that areas with a history of grazing supported higher chl *a*-biomass than the never-grazed areas (Zero grazing inside, overall mean:  $2.0 \pm 0.38 \mu\text{g chl } a \text{ cm}^{-2}$ ; Zero grazing outside:  $1.6 \pm 0.44 \mu\text{g chl } a \text{ cm}^{-2}$ ; InOut effect  $F_{1,11} = 12.04$ ,  $p = 0.005$ ). Difference in chl *a* inside to outside halos did not vary by month of observation or by snail density (InOut  $\times$  Month and InOut  $\times$  Density:  $p > 0.5$ ).

Table 2. Response of halo area as a proportion of pre-treatment area to 3 levels of original snail density (4, 8–11 or 17–21 snails halo<sup>-1</sup>), 3 levels of snail Grazing Intensity (Zero  $G_0$ , Half  $G_{0.5}$  or Full  $G_1$  grazing intensity, generated by removing 100, 50 or 0% of original snail numbers per halo) and 5 sampling Months (November and December 2007, January, February, and April 2008). Orthogonal contrasts are shown (indented) partitioning sums of squares of Grazing Intensity between a contrast of ungrazed ( $G_0$ ) to grazed ( $G_{0.5}$  and  $G_1$  pooled) and a contrast of grazed regimes ( $G_{0.5}$  vs.  $G_1$ ). **Bold**: significant; Seq: sequential; Adj: adjusted

Effect	df	Seq. SS	Adj. SS	Adj. MS	F	p
<b>Between halos</b>						
Grazer Density	2	0.068	0.068	0.033	0.19	0.825
Grazing Intensity	2	2.145	2.145	1.073	6.13	<b>0.005</b>
$G_0$ ungrazed vs. Grazed	1	2.046	2.046	2.046	11.71	<b>0.002</b>
$G_{0.5}$ vs. $G_1$ Grazing	1	0.100	0.100	0.100	0.57	0.455
Density $\times$ Intensity	4	0.360	0.360	0.090	0.51	0.73
Halo (Density $\times$ Intensity)	34	5.941	5.941	0.175	–	–
<b>Within halos</b>						
Month	4	1.720	1.720	0.430	15.40	<b>&lt;0.001</b>
Month $\times$ Density	8	0.276	0.282	0.035	1.26	0.269
Month $\times$ Intensity	8	1.311	1.311	0.164	5.87	<b>&lt;0.001</b>
Month $\times$ ( $G_0$ vs. Grazed)	4	1.247	1.247	0.312	11.16	<b>&lt;0.001</b>
Month $\times$ ( $G_{0.5}$ vs. $G_1$ )	4	0.064	0.064	0.016	0.57	0.683
Month $\times$ Density $\times$ Intensity	16	0.554	0.554	0.035	1.24	0.245
Month $\times$ Halo (Density $\times$ Intensity)	134	3.741	3.741	0.028		

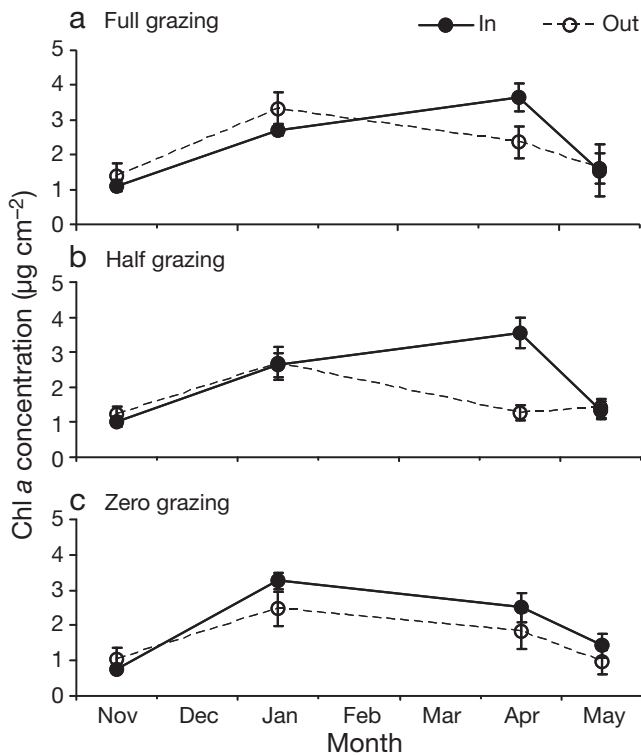


Fig. 5. Mean  $\pm$  SE chl *a* concentration inside and outside grazing halos with Full, Half and Zero grazing intensity, from November 2007 to May 2008

## DISCUSSION

Grazing halos of *Melarhaphes neritoides* generally diminished in area from autumn to spring. This reduction parallels the characteristically high growth of biofilms and ephemeral macroalgae over the winter period (Hawkins & Hartnoll 1983a, MacLulich 1987,

Hill & Hawkins 1991, Thompson et al. 2000, 2004). Experimental removal of snails further increased halo shrinkage. After removal (7 mo), halos with 50% removal had ~50% reduction in area, and those with 100% removal had almost completely disappeared. These observations show grazing controlled biofilm cover in areas near to refuges. This finding was expected: grazer removal from sections of rocky shores generally leads to a rapid increase in algal cover (Hawkins & Hartnoll 1983b, Jenkins et al. 2001, Coleman et al. 2006). Stafford & Davies (2005) found the exclusion of *M. neritoides* from fenced areas led to a reduced number of grazing halos. Halo shrinkage implies that snails reduced their foraging distance from refuges, and/or that biofilm growth from edges exceeded biofilm removal by grazers. Snails might have reduced their foraging range because food abundance rose over winter, as evidenced by steadily rising chl *a* concentrations inside grazed halos. This may be because their metabolic demands and grazing activity generally diminish with the winter decrease in temperature (Thompson et al. 2000, 2004). Seasonal rise in chl *a* inside halos could result from reduced grazing activity, and/or from upward growth from biofilm reservoirs in micropits within halos (Fig. 2d). Biofilm recruitment from water was probably less important at these sites in the splash zone above the tidal reach.

Biofilms with a history of snail grazing had significantly higher chl *a* biomass than areas without: chl *a* was higher inside halos with all snails removed than outside. Effect of grazing history was still evident at termination of the experiment, 7 mo after snail removal, suggesting an enduring effect of grazing. Painter et al. (1993) found the effect of prairie dog and

Table 3. Response of chl *a* concentration within grazing halos to original snail Density (4, 8–11, 17–21 snails halo<sup>-1</sup>), Grazing Intensity (Zero G<sub>0</sub>, Half G<sub>0.5</sub> or Full G<sub>1</sub> grazing) and Month (November 2007, January, April, and May 2008). Response tested was ln-transformed difference between µg chl *a* cm<sup>-2</sup> inside to outside each halo [ $\ln(\text{chl } a_{\text{in}}) - \ln(\text{chl } a_{\text{out}})$ ]. Orthogonal contrasts (indented) as for Table 2. **Bold**: significant; Seq: sequential; Adj: adjusted

Effect	df	Seq. SS	Adj. SS	Adj. MS	F	p
<b>Between halos</b>						
Grazer Density	2	5.617	5.422	2.711	0.88	0.423
Grazing Intensity	2	4.891	4.891	2.446	0.80	0.459
G <sub>0</sub> ungrazed vs. Grazed	1	0.475	0.475	0.475	0.15	0.697
G <sub>0.5</sub> vs. G <sub>1</sub> Grazing	1	4.417	4.417	4.417	1.44	0.239
Density $\times$ Intensity	4	5.892	5.892	1.473	0.48	0.750
Halo (Density $\times$ Intensity)	34	104.337	104.337	3.069	–	–
<b>Within halos</b>						
Month	3	73.136	73.136	24.379	21.30	<b>&lt;0.001</b>
Month $\times$ Density	6	7.386	7.518	1.253	1.09	0.371
Month $\times$ Intensity	6	30.765	30.765	5.128	4.48	<b>&lt;0.001</b>
Month $\times$ (G <sub>0</sub> vs. Grazed)	3	26.224	26.224	8.741	7.64	<b>&lt;0.001</b>
Month $\times$ (G <sub>0.5</sub> vs. G <sub>1</sub> )	3	4.541	4.541	1.514	1.32	0.271
Month $\times$ Density $\times$ Intensity	12	10.104	10.104	0.842	0.74	0.714
Month $\times$ Halo (Density $\times$ Intensity)	102	116.753	116.753	1.145		

ungulate grazing was still evident in the composition of North American grass lands >30 yr after grazer removal. Our experiments show that effects of grazing history are not exclusive to terrestrial macrophytic systems.

Evidence of grazing history is typically ascribed to grazer-instigated shifts in the composition or structural organisation of plant communities (Kaehler & Froneman 2002, McIntire & Hik 2005). The biofilm community could not be sampled in ungrazed halos because of the destructive nature of SEM and HPLC, and therefore we cannot know how the community responded to snail removal. SEM samples did show, however, that never-grazed areas outside halos had a thick carpet of EPS, detritus and dominant filamentous cyanobacteria, which in grazed areas inside halos was exchanged for abundant colonies of coccoid cyanobacteria and lichen. We suspect that these grazing-induced changes in biofilm community persisted after grazer removal and that they might have impacted on chl *a* biomass in 2 principal ways: (1) grazing may have boosted biofilm chl *a* biomass by suppressing the growth of competitively dominant biofilm species with low productivity (Norton et al. 1990, Kaehler & Froneman 2002); (2) grazing may have removed a canopy of largely dead biofilm, facilitating light and nutrient penetration for a faster growing community (Hillebrand et al. 2000, Kaehler & Froneman 2002).

Explanation (1) has no direct support, since productivity was not measured. However, never-grazed areas did have proportionally more filamentous cyanobacteria, while grazed areas had more coccoid cyanobacteria and lichen. Cell chains and filamentous growth forms are generally absent from grazed biofilms because larger and filamentous taxa are more easily removed by grazers (Nicotri 1977, Norton et al. 1990, Kaehler & Froneman 2002). Studies from a wide range of macrophytic systems have likewise found that community responses to grazing are associated with plant growth form (Noy-Meir et al. 1989, Huntly 1991). Explanation (2) is well supported by SEM observations. The thick carpet of EPS and detritus that dominated never-grazed areas may well have prevented light and nutrient penetration for biofilm growth. Such desiccated biofilm can linger for months after cell death in never-grazed areas at the study shore. Observed elevation in chl *a* biomass in areas with grazing history is thus plausibly explained by a lingering effect of snails grazing away overlaying detrital material and facilitating new production by the biofilm.

Against expectations, biofilm chl *a* biomass did not increase following grazer removal, irrespective of original grazer density and level of grazer removal. Instead, grazed biofilms reached higher chl *a* peaks

than never-grazed and in spring there was double the amount of chl *a* inside grazed halos than outside (Fig. 5). The biomass of terrestrial and freshwater macrophytic communities is generally negatively impacted by grazing, certainly at levels above low grazer density (Huntley 1991, McIntire & Hik 2005, Hillebrand 2008) and previous studies of rocky shores biofilms corroborate this (Williams et al. 2000, Stafford & Davies 2005, Underwood & Murphy 2008). The explanations above for grazing history boosting biofilm biomass may equally apply to areas exposed to grazing. Intertidal snails can additionally fertilise micro-algae by excretion of limiting nutrients (Plagányi & Branch 2000) and mucus deposition can stimulate micro-algal attachment and growth (Davies et al. 1992). Williams et al. (2000), however, found a negligible effect of snail mucus deposition in comparison to the increase in biomass that resulted from snail exclusion.

The use of chl *a* as a proxy of biofilm biomass is standard in microalgal and biofilm studies where it is not practicable to measure all biomass components (Thompson et al. 1999, Murphy et al. 2006). Our chl *a*-based results show that grazers boosted the biomass of living—i.e. chl *a* containing—micro-autotrophs. Biofilms additionally include materials without chl *a*, such as EPS, detritus and heterotrophic bacteria (Bärlocher & Murdoch 1989, Decho 2000). The biomass of all biofilm components combined was probably higher in ungrazed areas than in grazed areas, due to the non-removal of biofilm detritus in the absence of grazing.

The non-invasive technique of CIR used in the present study estimates biofilm chl *a* biomass from the remote sensing index NDVI (Murphy et al. 2006). NDVI relies on detecting the light absorbance-reflection by chl *a* (Murphy et al. 2006). However, light might not reach all the underlying chl *a* of a thick biofilm. CIR can therefore under-estimate chl *a* in rich biofilms. The risk of underestimation becomes significant in biofilms with chl *a* concentrations >10  $\mu\text{g cm}^{-2}$  (Buschmann & Nagel 1993, Meleder et al. 2003, Murphy et al. 2006). All CIR observations here were of chl *a* <7.4  $\mu\text{g cm}^{-2}$ , with the great majority being <3  $\mu\text{g chl } a \text{ cm}^{-2}$ . We therefore consider it unlikely that chl *a* was disproportionately underestimated in our ungrazed (thick) biofilms.

The fine-scale ( $\leq 5$  mm) distribution of chl *a* was more variable inside than outside halos. Although this might suggest grazers foraged unevenly, the patchiness inside halos was more likely caused by the uneven distribution of substratum micro-pits, which provided micro-organisms with refuges from grazing (Fig. 2). Hutchinson et al. (2006) found that the small-scale patchiness and species composition of grazed biofilms depended on the pittedness of artificial colonisation surfaces.

Natural snail density per grazing halo had no effect on the area grazed per snail, indicating an absence of facilitation or interference between snails in the exploitation of their biofilm food. Competition in food exploitation is common among intertidal snails, although the strength of effect is often weak or entirely absent (reviewed by: McQuaid 1996). There have been no studies of competition in *Melarhaphé neritoides*, although work has been done on similar high shore littorinids (e.g. Branch & Branch 1981). The lack of evidence for interference found here might suggest that food was plentiful over the observation period. This is supported by the observation that area grazed per snail dropped steadily over winter as the biofilm concentration in foraging halos rose. Periwinkle snails do regulate foraging area to suit requirements. Sommer (1999) found laboratory cultures of low shore periwinkle *Littorina littorea* increased the area grazed when food was sub-saturated. Our results show that *M. neritoides* compensated for increased densities in halos by enlarging foraging range, thereby keeping a constant grazing area per snail. This regulation will function up until a threshold level of density, above which the foraging range becomes energetically unsustainable and risky (due to dislodgement, predation, desiccation). Unless mortality and immigration prevent *M. neritoides* from reaching this threshold density, a reduction in grazing area per snail should be detectable at very high densities.

### CONCLUSIONS

The present study has shown that a history of grazing by *Melarhaphé neritoides* boosted biofilm chl *a*. Grazing did not reduce chl *a* to a level lower than that of never-grazed areas, even at high snail density, and in spring there was a trend for the chl *a* in grazed areas to exceed that in ungrazed areas with a history of grazing. Biomass removed by grazing was probably compensated for by increased production stimulated by releasing the photosynthesising biofilm from being covered by a partly dead and unproductive biofilm. It is not known if grazing-induced increase in chlorophyll biomass also translated into a rise in photosynthetic production.

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