Vol. 608: 119–132, 2019
https://doi.org/10.3354/meps12785

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published January 3

# Seasonal changes in bacteria and phytoplankton biomass control the condition index of the demosponge *Halichondria panicea* in temperate Danish waters

**Florian Lüskow[1,3,*], Hans Ulrik Riisgård[1], Vita Solovyeva[2], Jonathan R. Brewer[2]**

[1]Marine Biological Research Centre, Department of Biology, University of Southern Denmark, Hindsholmvej 11, 5300 Kerteminde, Denmark

[2]Danish Molecular Biomedical Imaging Center (DaMBIC), Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark

[3]*Present address:* Department of Earth, Ocean and Atmospheric Sciences, University of British Columbia, Vancouver, 2039-2207 Main Mall, British Columbia V6T 1Z4, Canada

ABSTRACT: Sponges are sessile filter-feeders, and the basic principles for water pumping and particle retention are the same among all demosponges. Phytoplankton cells smaller than the inhalant openings (ostia) are retained and phagocytosed in the inhalant canal system before the water is sieved through the collar filter of the choanocytes, which efficiently retain bacteria. However, the importance of bacteria versus phytoplankton as the main diet is unclear. Sponges must cope with seasonal changes in food availability in temperate waters, and during winter, low phytoplankton and bacterial biomasses may result in starvation. In this study, the lower threshold of suspended biomass resulting in starvation of the demosponge *Halichondria panicea* was determined. We measured the seasonal changes in phytoplankton and bacterial biomasses along with the sponge condition index (CI, the ratio of organic to inorganic matter). A low CI during winter reflected starvation, and based on total available carbon concentrations (phytoplankton plus bacterial carbon), we conclude that a concentration of ca. 30 µg C $l^{-1}$ was not sufficient to cover the maintenance costs of *H. panicea*. Bacteria constitute a minor, but consistent part of the diet, around 20%. A field growth experiment revealed a positive correlation between CI and sponge weight. Measured volume-specific clearance rate of sponges, density of choanocyte chambers and inter-choanocyte chamber-distance were not significantly different in low and high CI sponges, and it remains unknown how sponges withstand long periods with low suspended food particle concentrations.

KEY WORDS: Porifera · Clearance rate · Free-living bacteria · Chlorophyll *a* · Starvation · Growth · Choanocyte chambers



## 1. INTRODUCTION

Sponges are sessile filter-feeders, and the basic principles for water pumping and particle retention are the same among all demosponges (Bergquist 1978, Larsen & Riisgård 1994, Riisgård & Larsen 2010, Leys et al. 2011). Water enters the sponge through numerous small openings (ostia) on the surface (exopinacoderm) and flows through a branched inhalant canal system to the water pumping units, the choanocyte chambers, each containing about 50 to 139 choanocytes (Ludeman et al. 2017). Every choanocyte has a beating flagellum and a collar of microvilli acting as a filter that allows capture of free-

*Corresponding author: flueskow@eoas.ubc.ca

living bacteria and other particles down to about 0.1 µm diameter (Fjerdingstad 1961, Reiswig 1975, Langenbruch 1983, Leys et al. 2011). Phytoplankton cells smaller than the ostia, with a diameter between 6.5 and 32 µm (Reiswig 1975), enter the ostia and are retained and phagocytosed in the extensive inhalant canal system (Kilian 1952) before the water is sieved through the collar filter of the choanocytes. The filtered water leaves the choanocyte chamber through an opening (apopyle) to the exhalant canal system that merges into one or more large openings (oscula) through which the water leaves the sponge in an exhalant jet (Bergquist 1978, Kumala et al. 2017). Larsen & Riisgård (1994) suggested that in a 'standard sponge', the choanocyte chambers, with a density of 12 000 chambers $mm^{-3}$ (Reiswig 1975), constitute 30 to 50% of the wall structure separating inhalant and exhalant canals, and thus stabilise the entire structure of the sponge.

Because sponges are extremely efficient in retaining free-living bacteria, but have an upper size limit for particles larger than the inhalant ostia, the importance of bacteria versus phytoplankton as their main diet is unclear, and therefore this question forms part of the present study. In temperate waters, filter-feeding sponges must cope with pronounced seasonal changes in food availability, i.e. free-living bacteria and phytoplankton (Smetacek 1985, Zweifel et al. 1993). During winter when light restricts primary production, phytoplankton biomass and production become low, and along with a subsequent decrease in heterotrophic bacterial biomass, may give rise to starvation of sponges. Bacterial density is otherwise mainly controlled by the grazing activity of flagellates limiting the bacterial concentration to about $1.5 \times 10^6$ cells $ml^{-1}$ (Azam et al. 1983, Fenchel 2008).

Starvation is considered a state in which energetic maintenance requirements cannot be matched anymore and may therefore be the major reason for sponge death *in vitro* (Arndt 1933). According to Barthel (1989), growth of the demosponge *H. panicea* in a temperate North Sea habitat varies strongly with season, with positive growth from June until October, whereupon the sponge starts to shrink in size. However, Barthel (1989) did not associate the limited food availability and starvation to shrinkage, but suggested that symbiotic algae and uptake of dissolved organic carbon (DOC) contribute to the nutrition during winter. For another temperate sponge species (*Haliclona oculata*), Koopmans & Wijffels (2008) found a similar trend, with lowest growth rate in January, and correlated the variation with differences in temperature, algal biomass and particulate organic matter (POM). In temperate Danish waters, blue mussels *Mytilus edulis* frequently co-occur with *H. panicea*, and both filter-feeders must cope with the same problem of shortage of food during winter months. While *H. panicea* stores almost no glycogen and relatively few lipids within its tissue (Barthel 1986), and has no specialised organs (Bergquist 1978, Simpson 1984) for storing energy reserves, *M. edulis* has well-developed glycogen reserves that can be metabolised during prolonged periods of starvation (Pleissner et al. 2012, Riisgård & Larsen 2015).

Koopmans et al. (2015) studied the fatty acid (FA) composition of 3 temperate sponge species (including *H. panicea*) in relation to seasonally changing POM concentration to quantify the contribution of bacteria, diatoms and dinoflagellates to their diets. They concluded that the overall FA concentration in sponges is related to the FA concentration in POM and therefore emphasized that the internal condition of the sponge is closely related to the ambient conditions, i.e. high body condition when ambient FA concentration in POM is elevated. *M. edulis* closes its valves, which reduces the respiration rate, when the phytoplankton biomass becomes too low to cover the mussel's metabolic demands (Riisgård et al. 2011, Tang & Riisgård 2016), but a comparable physiological mechanism has not been described for *H. panicea*. Barthel (1989) did not relate growth to food availability, but suggested that the water temperature was an important factor governing growth of *H. panicea*. Temperature was also found to be in a negative correlation with choanocyte chamber density, when experimentally increased beyond the natural range (Massaro et al. 2012). Using the *F/R*-ratio (litres of water filtered per ml of oxygen respired), Riisgård et al. (2016) evaluated the ability of *H. panicea* to feed solely on phytoplankton versus free-living bacteria. It was estimated that the content of suspended particulate organic carbon must be at least 0.03 mg C $l^{-1}$ to cover the maintenance requirement of *H. panicea*, which may not be available during winter (Riisgård et al. 2016). Thus, the sponge may be able to survive on a sole diet of phytoplankton in the productive season even when free-living bacteria are also accessible to the sponge, and bacteria possibly are an important although insufficient food source in terms of carbon content and concentration compared to phytoplankton.

The primary aim of the present study was to determine a lower biomass threshold resulting in incipient starvation of *H. panicea* indicated by a low condition index (CI, i.e. the ratio of organic to inorganic matter). This was done by following the seasonal

changes in available phytoplankton biomass and free-living bacteria concentration along with measurements of the sponge CI as a proxy for favourable or unfavourable growth conditions. The secondary goal was to test the hypothesis whether clearance rates of *H. panicea* are different at low (expected for winter) and high (expected for summer) CI, respectively, and explore if the filtration activity was affected by differences in density of choanocyte chambers. A field growth experiment conducted in spring and early summer aimed at correlating the CI with increasing sponge body weight. Thus, an important goal was to improve our present understanding of how sponges in temperate waters cope with winter starvation and recovery during spring and early summer.

## 2. MATERIALS AND METHODS

### 2.1. Collection of sponges and preparation of explants

*Halichondria panicea* specimens were regularly collected in the inlet to Kerteminde Fjord, Denmark (55° 26′ 59″ N, 10° 39′ 41″ E), between January 2016 and May 2017, cleaned of epifauna and placed in bio-filtered running seawater (temperature = 1.8–21.2°C, salinity = 8.9–29.5). Additionally, for feeding experiments, we collected larger specimens (sponge volume, $V_{sp}$ = 18–187 ml) from maximal 2 m depth and either directly transplanted into the open-top chamber setup (see 'Clearance measurements' below) or kept in a cage in the fjord for a maximum of 2 wk. For growth studies, entire sponges ($V_{sp}$ = 6.2–22.5 ml) were carefully detached from harbour walls (same population as used for CI calculations) and directly, without exposing them to air, brought into the nearby laboratory where they were each placed on PVC plates of around 36 $cm^2$ in running seawater aquaria. The specimens were fixed with a synthetic thread and allowed to attach to the substratum for up to 19 d before they were brought out into the fjord.

### 2.2. Environmental parameters and bacterial concentration

Hydrographic parameters (temperature, salinity and chlorophyll *a* [chl *a*] concentration) were measured every morning, during the course of 1 yr at 1 m depth (and 1 m away from the harbour wall) near the sponge collection site using a YSI 650 (Yellow Springs Instrument; 6 to 12% uncertainty of chl *a* measurements in the used range) or a handheld fluorometer (AquaFluor).

Water samples (5 ml) were taken once or twice a week at the same place to determine the bacterial concentration (in triplicate) using 4′-6′-diamidino-2-phenylindole (DAPI, Roche Diagnostics) staining and epifluorescence microscopy according to Porter & Feig (1980). Samples were preserved in 1.5 ml 1% glutaraldehyde and stored in a refrigerator until analysis. Samples were filtered through a 0.2 µm black polycarbonate sheet filter (Whatman Nuclepore Track Etch Membrane) and subsequently stained with 100 µl DAPI (working solution: 198 µg $ml^{-1}$) for at least 4 min. When used in combination with epifluorescence microscopy (Leica, type 020-505.030) and UV excitation (Leica Hg-lamp Osram HBO 50 W L2; filter cube A: excitation filter: Bandpass 340–380 nm, emission filter: Longpass 425 nm), 60 to 300 bacteria were identified during each count (1000× magnification) in accordance with Muthukrishnan et al. (2017).

Measured overall chl *a* concentrations, including phytoplankton species not available to the sponge, were converted to phytoplankton carbon biomass ($PPCB_{pre}$, µg C $l^{-1}$) using 1 µg chl *a* = 40 µg C (Li et al. 2010). The $PPCB_{pre}$ was corrected for species cell or cell colony size ($PPCB_{post}$, µg C $l^{-1}$, based on the species catalogue from NOVANA 2017), by excluding the carbon contribution of all species >32 µm (cf. Reiswig 1975, diameter of ostia in his Table 1), which was set to be the upper cell or cell colony size of phytoplankton entering the sponge. In the present study, phytoplankton composition data were used from 2011 (the only recent available data set for the adjacent Great Belt), which might not necessarily reflect the situation in 2016 and early 2017 (and respective total carbon biomass [TCB] development is mainly driven by the size distribution of phytoplankton, since only some sizes are able to enter the ostia). Fenchel (1982) determined carbon contents from laboratory-cultivated bacteria ($1 \times 10^{-4}$ µg C $cell^{-1}$), whereas Fukuda et al. (1998) determined the average carbon content for natural coastal bacterial assemblages to be $30.2 \times 10^{-9}$ µg C $cell^{-1}$. However, Ferguson & Rublee (1976) measured a substantially lower value for field-collected bacteria ($7.8 \times 10^{-9}$ µg C $cell^{-1}$), which we have used here as a conservative estimate, although the use of only 1 conversion factor might be an oversimplification of the bacterial community composition and its seasonal change. Based on the corrected phytoplankton biomass ($PPCB_{post}$) and the bacterial carbon biomass (BCB, µg C $l^{-1}$, $7.8 \times 10^{-9}$ µg C $cell^{-1}$, Ferguson & Rublee 1976), we calculated the total carbon biomass (TCB) available to the sponge as $PPCB_{post}$ + BCB.

### 2.3. Sponge size and CI

The growth and CI of sponges were determined by means of volume-specific dry weight ($DW_v$, mg (ml sponge)$^{-1}$; 100°C, 24 h) and ash-free dry weight ($AFDW_v$, mg (ml sponge)$^{-1}$; 500°C, 6 h, cf. Thomassen & Riisgård 1995) (Fig. 1). The sponge volume was determined by adding the sponge to a known amount of seawater and measuring the displaced volume. Sample weight was determined on a precision balance (Sartorius BP210D). Based on volume-specific weights, CI was calculated as the ratio of organic to inorganic matter (Barthel 1986):

$$CI = AFDW_v / (DW_v - AFDW_v) \quad (1)$$

### 2.4. Field growth experiments

Sponge explants were brought out to the inlet of Kerteminde Fjord in April 2016 and installed in frames at 1 m depth, which were held in position by means of buoys and anchors (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m608p119_supp/, explant initial wet weight $WW_0$ = 11.0 ± 5.6 g, n = 14). Every week the sponge explant holding plates were removed and brought to the nearby laboratory. During the entire recording process (approximately 30 min), sponges were only minimally exposed to air. Sponges and substrates were carefully cleared of epifauna, using cotton sticks, and their wet weights were measured every week using a precision balance (Sartorius LP12000S). Pre-determined plate weights allowed this procedure. Wet weight-specific growth rates ($\mu_{WW}$, % d$^{-1}$) were estimated from exponential regression analysis.

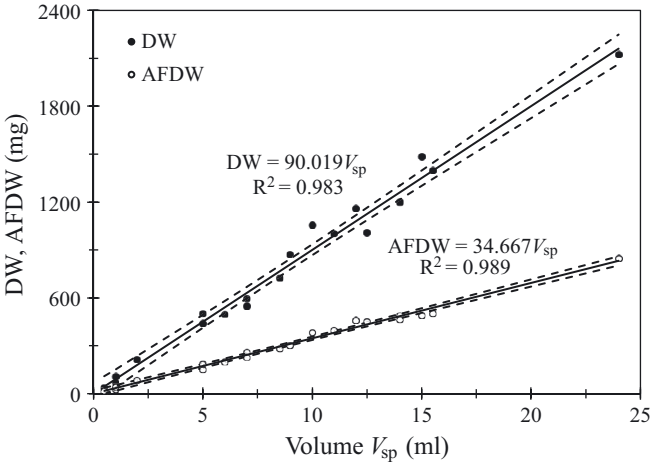



Fig. 1. Dry weight (DW, mg) and ash-free dry weight (AFDW, mg) as a function of sponge volume ($V_{sp}$, ml) of *Halichondria panicea* measured on 9 December 2016 (cf. no. 21 in Table S1 in the Supplement, n = 20, range 0.5–24 ml). Linear regression lines are shown along with their equations and 95% confidence intervals (dashed lines)

### 2.5. Clearance measurements

Measurements of clearance rates (volume of water cleared of particles per unit of time) were performed in an *in situ* setup consisting of a moveable bottom plate that can be vertically adjusted and sealed with an open-top chamber (cf. Lüskow & Riisgard 2018). This setup allowed the addition of algal suspension and removal of samples by means of a pipette in experiments in autumn (n = 8) and winter (n = 9). Sponges were placed in groups ($V_{sp}$ = 80–426 ml; Fig. 2) on the movable bottom plate and were allowed to acclimate for 15 min. The transparent open plastic cylinder was placed on top of the sponge holding plate and carefully screwed at the bottom (remaining volume in the chamber was between 6.9 and 29.1 l in all experiments). Two air stones at the periphery of the tube allowed sufficient water mixing without disturbing the sponges. By taking water samples (25 ml) in discrete time intervals from the mid-volume (approximately 10 cm above the sponges), the decrease in relative algal concentration ($Conc_{alg}$) as a function of time was recorded over a period of 30 min. When $Conc_{alg}$ fell below a certain level (reduction of about 50%), new algae (*Rhodomonas salina*) were added to the cylindrical tube. Cell concentrations (cells ml$^{-1}$) were determined from water samples preserved with 2.5 ml Lugol's solution (6% iodine-potassium, 4% iodine solution) using Utermöhl's cell counting technique and tubular counting chambers (KC Denmark)

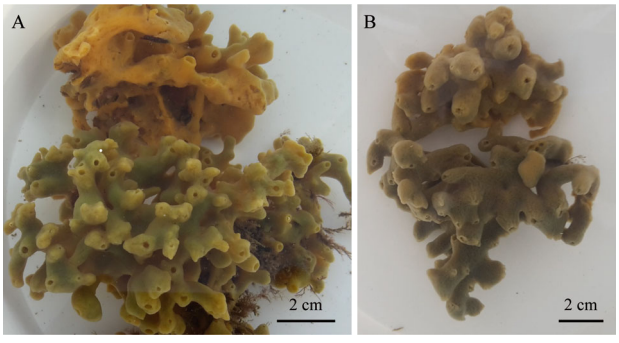



Fig. 2. *Halichondria panicea* specimens photographed after being used in *in situ* clearance experiments on (A) 22 September 2016 and (B) 14 February 2017. Size ranges of sponges used in September and February were similar. Sponges in different seasons look healthy from the outside, with no degenerated parts

under an inverted microscope (Leica, DMIRB, 200× magnification). The relative algal concentration was determined by counting *R. salina* cells (>100 cells) within a known number of fields of view (>6 fields) so that the relative cell concentration was expressed as cells field$^{-1}$. Following the exponential decrease in $Conc_{alg}$ over time, the volume-specific clearance rate ($CR_v$, ml water (ml sponge)$^{-1}$ min$^{-1}$) of a sponge was calculated as per Riisgård et al. (2016):

$$CR_v = V \times b / V_{sp} \quad (2)$$

where $V$ = volume of seawater in the chamber, $b$ = slope of regression line in a semi-ln plot for the reduction in algal concentration versus time and $V_{sp}$ = sponge volume. Daily controls with *R. salina* (and without sponges) showed only small reductions in the algal concentration over time, which were subtracted from sponge clearance rates.

### 2.6. Choanocyte chamber density

The volume-specific number of choanocyte chambers (density, $D$, in chambers mm$^{-3}$) was comparatively evaluated in sponges from the inlet to Kerteminde Fjord taken during winter (i.e. January) and spring (i.e. May). Freshly collected sponges were placed in buckets with seawater and transported to the University of Southern Denmark within 3 h after sampling. These sponges (n = 6) with numerous exhalant openings were separately placed in 20 ml beakers filled with 200 µm filtered seawater from Kerteminde Fjord. Samples were stained with 100 µl (working solution: 10 µg ml$^{-1}$) green polystyrene nanobead stock solution (G50, Fluoro-Max; diameter 47 nm, 468 nm excitation/508 nm emission) during an ingestion time of 24 h in the dark and at room temperature. The next day, 3 distinct chimneys of each sponge (n = 6) were cut off with a scalpel, coated with optimum cutting temperature mounting medium for cryotomy, placed in an aluminium container and frozen in isopentane chilled with liquid nitrogen. Samples were stored at –20°C until analysis. Mounted sponge chimneys were cut at about mid-height into 30 µm slices using a cryo-cutter (Shandon Cryotome FSE, Thermo Fisher Scientific), washed with phosphate-buffered saline and mounted with ProLong Diamond antifade reagent on a glass slide. The prepared chimney cross sections were imaged using a Zeiss LSM 510 META laser scanning microscope using a 63× water objective with a numerical aperture of 1.2 and a 488 nm excitation laser (Argon/2), operated by LSM 4.2 software (Sequence S1 in the Supplement). In the case of the confocal images taken, the resolution is below 0.3 µm in $x$ and $y$ planes and on the order of 0.5 µm in the $z$ plane (Pawley 2006). Randomly selected spots were imaged using $z$-stacking (width: 450 µm, length: 450 µm, depth: 8–25 µm, layers in 1 µm intervals). Choanocyte chambers were identified from the $z$-stack (image recording of sample volume at different focal planes) image sequences using the image analysis software ImageJ (version 1.48v; Rueden et al. 2017). Using ImageJ, chamber positions ($x$, $y$ and $z$ coordinates) were measured. The coordinates of the chamber centre were determined at the maximum diameter in the image sequence. This enables measurements of chamber density and inter-chamber distance in 3 dimensions in the intact 30 µm sample section. Based on choanocyte chamber counts and $z$-stack dimensions, volume-specific densities ($D$, chambers mm$^{-3}$) were calculated. All distances ($d$) from 1 chamber centre to all others within 1 sample were calculated using Eq. (3):

$$d = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2} \quad (3)$$

where $x_1$, $y_1$ and $z_1$ denote the coordinates of chamber centre 1 and $x_2$, $y_2$ and $z_2$ are the coordinates of chamber centre 2.

The shortest distance was listed per chamber using C++. Finally, the mean (µm ± SD) of all shortest distances within 1 sample was calculated and compared between high and low condition sponges.

### 2.7. Statistical analyses

One-way ANOVA corrected for sampling day was used to investigate differences in CI of sponges, temperature, salinity and chl *a* in experiments performed in autumn (n = 8) and winter (n = 9) 2016. Temporal variation in the DW:WW ratio was tested by a 1-way ANOVA, followed by a Tukey-HSD post hoc test in order to differentiate between seasons. One-way ANOVA was further used to analyse differences in volume-specific clearance rate and choanocyte chamber densities of low and high condition sponges. Analysis of covariance (ANCOVA) was applied for the investigation of interactive effects of temperature and chl *a* on clearance rates measured and CI calculated in autumn and winter. Residuals have been tested for homogeneity. All statistical tests were performed in R (R Core Team 2017). The hypothesis of significance was accepted for $p < 0.05$.

## 3. RESULTS

### 3.1. Seasonal variation in environmental parameters and food concentration

Environmental parameters (temperature, salinity, chl *a*) and bacterial concentrations were generally measured on a daily basis between January 2016 and January 2017 (Fig. 3A,B). Chl *a* (µg $l^{-1}$, as an indicator for overall phytoplankton density) and bacterial concentrations ($Conc_{bac}$, cells $ml^{-1}$) were subject to pronounced seasonal variations (Fig. 3B). Winter concentrations of 2.0 ± 0.4 µg $l^{-1}$ chl *a* and 0.3 ± 0.1 × $10^6$ bacteria $ml^{-1}$ (mean ± SD from January to February 2016) increased rapidly during March, to 3.2 ± 0.9 µg $l^{-1}$ chl *a*, although the increase in bacterial concentration to 0.8 ± 0.2 × $10^6$ bacteria $ml^{-1}$ was somewhat delayed (Table 1). After this peak, concentrations stayed high until the beginning of December (3.1 ± 1.2 µg $l^{-1}$ chl *a* and 0.8 ± 0.2 × $10^6$ bacteria $ml^{-1}$). In the following winter, chl *a* and bacterial concentration steadily decreased and reached 1.5 ± 0.9 µg $l^{-1}$ chl *a* and 0.4 ± 0.2 × $10^6$ bacteria $ml^{-1}$ in January 2017. The phytoplankton biomass (measured as chl *a*) varied during the season, with low concentrations during winter (Fig. 3B). Using the same data, the relative importance of bacteria in the sponges' diet also varied during the season, with lowest values in the winter period, when primary production—and thus loss of dissolved organic matter from phytoplankton to be taken up by heterotrophic bacteria—was also lowest (Fig. S2 in the Supplement).

Carbon available to the sponge via uptake of phytoplankton and bacteria was calculated on the basis of measured concentrations (Fig. 3C). While the bacterial concentration could be directly converted to bacterial carbon biomass (BCB, µg C $l^{-1}$), the chl *a* (as a proxy for phytoplankton biomass, pre-corrected) first had to be corrected for species-specific contributions to the overall phytoplankton biomass (post-corrected). Therefore, the pre-corrected phytoplankton biomass ($PPCB_{pre}$) can be understood as a direct conversion of chl *a* to carbon concentration. The corrected phytoplankton biomass ($PPCB_{post}$) and bacterial carbon biomass (BCB) add up to the total sponge-available carbon (TCB, µg C $l^{-1}$) as shown in Fig. 3C. The contribution of BCB to TCB is rather small year-round (17.8 ± 12.9%), while the overall biomass trend is defined by the development of the corrected phytoplankton biomass (Table 1). The total biomass increased from a winter minimum of 7.5 µg C $l^{-1}$ on 4 February 2016 to a spring maximum of 153.8 µg C $l^{-1}$ on 30 March 2016. The spring–early summer peak (108.7 µg C $l^{-1}$) steadily vanished throughout summer (71.5 µg C $l^{-1}$) and autumn (31.0 µg C $l^{-1}$), while in winter 2017, only 27.2 µg C $l^{-1}$ were available to the sponges (calculated mean concentrations per season, Fig. 3C, Table 1).

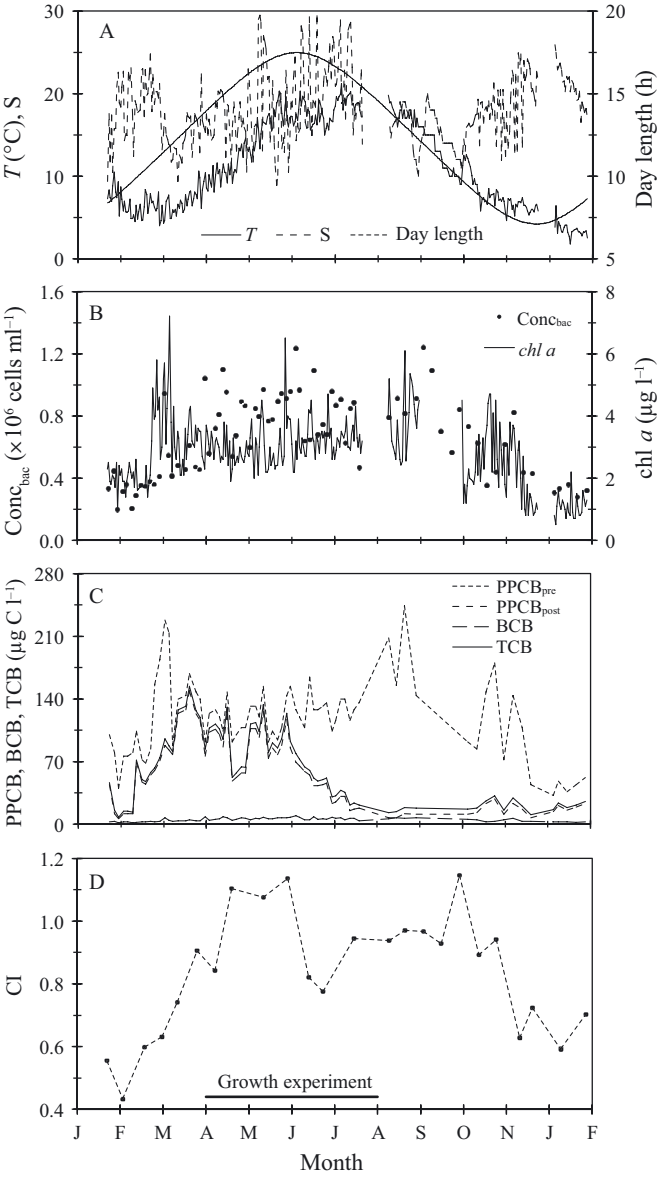



Fig. 3. Environmental parameters as a function of time (months) measured every day mainly between 09:00 and 12:00 h during 2016 and beginning 2017 at 1 m depth in the inlet to Kerteminde Fjord: (A) *T*: temperature (°C), S: salinity, and day length (h, time between sunrise and sunset, from www.sunrise-and-sunset.com). (B) Phytoplankton (measured as chlorophyll *a* concentration in µg $l^{-1}$) and mean bacterial concentration ($Conc_{bac}$, ×$10^6$ cells $ml^{-1}$). (C) Phytoplankton (PPCB, pre- and post-corrected), bacteria (BCB) and total carbon biomass (TCB, µg C $l^{-1}$). (D) Condition index (CI). Indices are calculated on the basis of a 'standard sponge' with a volume of 10 ml using $DW_v$ and $AFDW_v$ from Fig. 1 and Table S1 in the Supplement. Bar indicates the duration of the growth experiment

Table 1. Seasonal development (winter 2015/2016 until winter 2016/2017) of environmental parameters; *T*: temperature, S: salinity, chl *a*: chlorophyll *a* concentration, $Conc_{bac}$: bacterial concentration. Mean phytoplankton ($PPCB_{post}$) and bacterial carbon biomass (BCB) are summed up to total sponge-available carbon biomass (TCB = $PPCB_{post}$ + BCB) and the BCB:TCB ratio. Data from Fig. 3. Mean ± SD are indicated

| Season | Period | *T* (°C) | S | chl *a* (µg $l^{-1}$) (min–max) | $PPCB_{post}$ (µg C $l^{-1}$) | $Conc_{bac}$ (×$10^6$ cells $ml^{-1}$) | BCB (µg C $l^{-1}$) | BCB/TCB (%) | TCB (µg C $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| Winter | Jan–Feb | 6.7 ± 1.5 | 18.5 ± 3.9 | 2.0 ± 0.4 (1.0–2.6) | 42.6 ± 22.0 | 0.3 ± 0.1 | 2.5 ± 0.6 | 11.5 | 45.1 |
| Spring | Mar–May | 9.8 ± 3.6 | 17.0 ± 4.1 | 3.2 ± 0.9 (1.9–7.2) | 103.3 ± 23.3 | 0.7 ± 0.2 | 5.4 ± 1.7 | 5.9 | 108.7 |
| Summer | Jun–Aug | 17.9 ± 1.4 | 19.8 ± 4.9 | 3.3 ± 0.7 (2.2–6.5) | 65.0 ± 23.3 | 0.8 ± 0.2 | 6.5 ± 1.4 | 15.5 | 71.5 |
| Autumn | Sep–Nov | 12.1 ± 4.1 | 16.0 ± 2.6 | 3.1 ± 1.2 (1.2–6.1) | 24.4 ± 5.8 | 0.8 ± 0.2 | 6.6 ± 0.7 | 39.6 | 31.0 |
| Winter | Dec–Jan | 4.8 ± 1.6 | 20.8 ± 3.2 | 1.5 ± 0.9 (0.5–3.8) | 24.0 ± 12.4 | 0.4 ± 0.2 | 3.2 ± 1.4 | 16.4 | 27.2 |
| | | | | | | | Mean ± SD | 17.8 ± 12.9 | |

### 3.2. Sponge CI

The CI (ratio of organic to inorganic matter) development over the 1 yr period is depicted in Fig. 3D (data from Tables S1 & S2). From a low CI in winter 2016 (0.53 ± 0.09) it increased during March and April along with the total carbon biomass (from 45.1 µg C $l^{-1}$ during winter to 108.7 µg C $l^{-1}$ in April) and peaked in May and June at CI = 1.14 (spring CI: 0.88 ± 0.19), when food conditions were high (Fig. 3C). After 1 mo, the CI dropped again to a summer minimum of 0.78 in July (summer CI: 0.92 ± 0.14) and recovered in August and September to an intermediate level (0.94–0.97). In the same period, the TCB gradually diminished to about 31.0 µg C $l^{-1}$ during late summer and autumn. After a short increase to 1.15 in October, the CI steadily decreased towards winter 2017 (autumn CI: 0.97 ± 0.09). During winter, the CI stayed as low as 0.66 ± 0.06, while the TCB was further diminished to 27.2 µg C $l^{-1}$ in this period (Table 1). The fraction of dry weight in wet sponge material (8.1 ± 0.9% dry weight share) varied throughout the seasons (Fig. 4), but changes were insignificant between seasons (ANOVA, $F_{4,19} = 1.270$, $p > 0.317$).

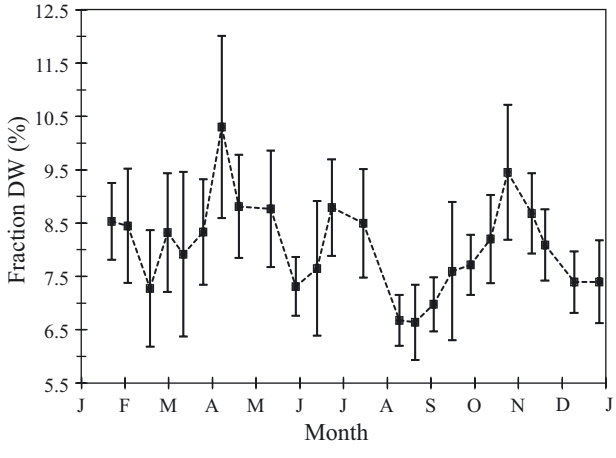



Fig. 4. Dry weight (mean ± SD) of *Halichondria panicea* as a fraction of wet weight (%) during 2016 and beginning 2017 in the inlet to Kerteminde Fjord

### 3.3. Field growth of sponges

A growth experiment on explanted sponges (initial wet weight, $WW_0$ = 11.0 ± 5.6 g, n = 14) was conducted between April and August 2016 (over a period of 125 d). The development of mean wet weight over time is shown in Fig. 5 (period indicated in Fig. 3D). The equation of an exponential regression line shows that sponge explants developed with a wet weight-specific growth rate ($\mu_{WW}$) of 0.6% $d^{-1}$.

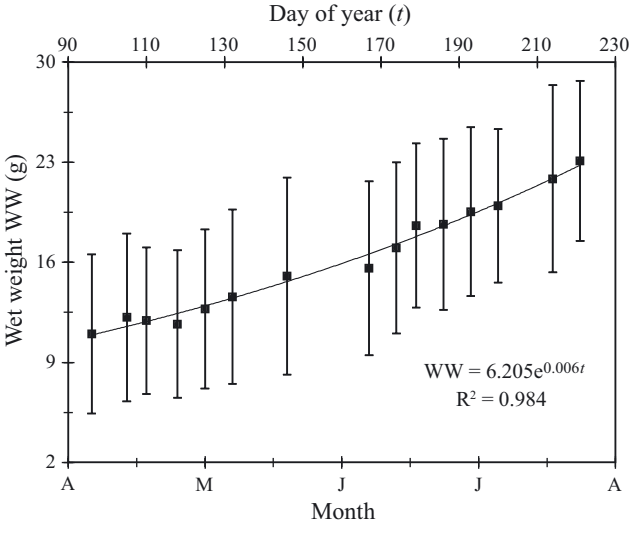



Fig. 5. Development of mean ± SD wet weight (WW, g) of *Halichondria panicea* explants (n = 14) at 1 m depth in the inlet to Kerteminde Fjord as a function of day of year (*t*). Exponential regression line is shown along with its equation. Exponent represents weight-specific growth rate

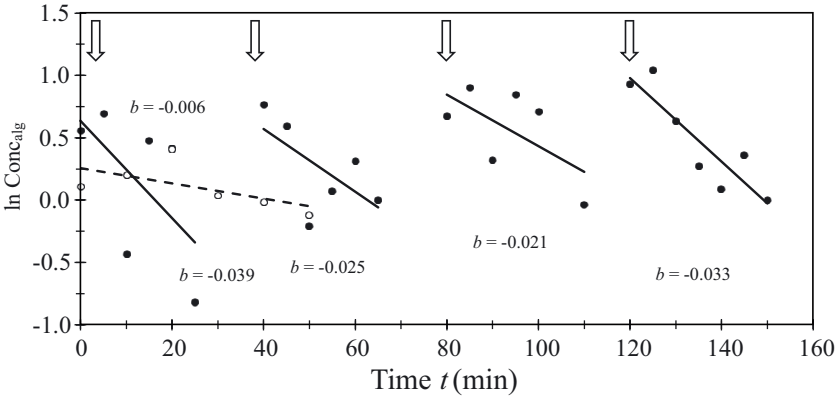



Fig. 6. Semi-ln plot of relative *Rhodomonas salina* concentration ($Conc_{alg}$) as a function of time in clearance experiments with repeated algal addition (arrows) in an *in situ* chamber with 4 *Halichondria panicea* colonies. Open circles (with dashed line) represent a control (i.e. without sponges). Linear regression lines are shown. $b$ = slopes of regression lines of decreasing algal concentration over time

### 3.4. Clearance rates, CI of sponges and dependency on environmental parameters

Repeated measurements of clearance rate from new algae addition are shown in Fig. 6. No significant difference between volume-specific clearance rates of sponges with low and high condition (autumn: 1.5 ± 0.8 ml water (ml sponge)$^{-1}$ min$^{-1}$, winter: 1.9 ± 1.1 ml water (ml sponge)$^{-1}$ min$^{-1}$) was detected (Fig. 7, ANOVA, $F_{1,15}$ = 0.7, p = 0.416). Environmental factors and CI were measured during experimental days, and we found that the temperature (ANOVA, $F_{1,10}$ = 159.5, p = 1.8 × 10$^{-7}$), chl *a* (ANOVA, $F_{1,15}$ = 35.449, p = 2.644 × 10$^{-5}$) and CI (ANOVA, $F_{1,10}$ = 5.808, p = 0.037) varied significantly between the experimental periods in autumn and winter, while the salinity was not significantly different (ANOVA, $F_{1,10}$ = 2.106, p = 0.177) between clearance experiments performed in autumn and winter (Fig. 8). Clearance rates of seasonally acclimated sponges did not vary significantly with respect to temperature and chl *a* (ANCOVA, $F_{1,8}$ = 0.1, p > 0.497). Temperature (ANOVA, $F_{1,8}$ = 8.5, p = 0.019) significantly affected the CI, while chl *a* (ANOVA, $F_{1,10}$ = 4.0, p = 0.073) was only marginally affected. There was no significant interaction of temperature and chl *a* on CI (ANCOVA, $F_{1,8}$ = 0.1, p = 0.723).

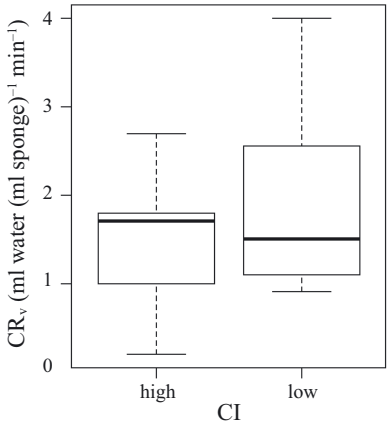



Fig. 7. Volume-specific clearance rates ($CR_v$) of *Halichondria panicea* fed with *Rhodomonas salina* with high condition index (CI = 0.94 ± 0.17, i.e. autumn; n = 8) and low CI (0.75 ± 0.10, i.e. during winter; n = 9). Central boxes show data between the 25$^{th}$ and the 75$^{th}$ percentiles, with the median represented as a line. The whiskers extend as far as the minimum and maximum values

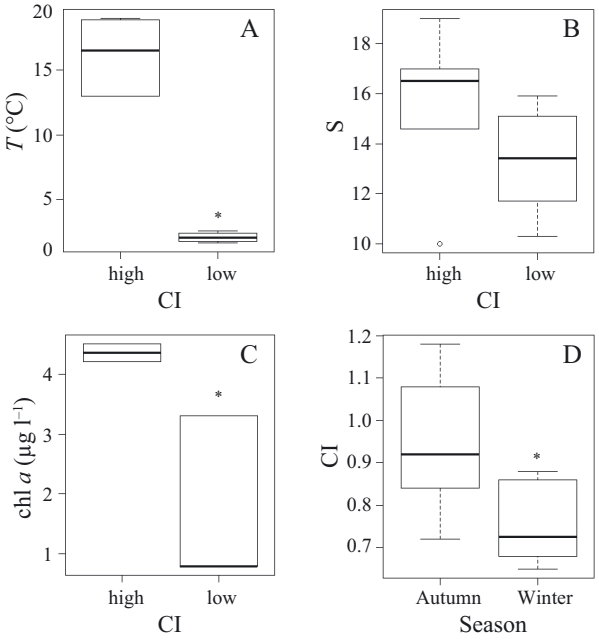
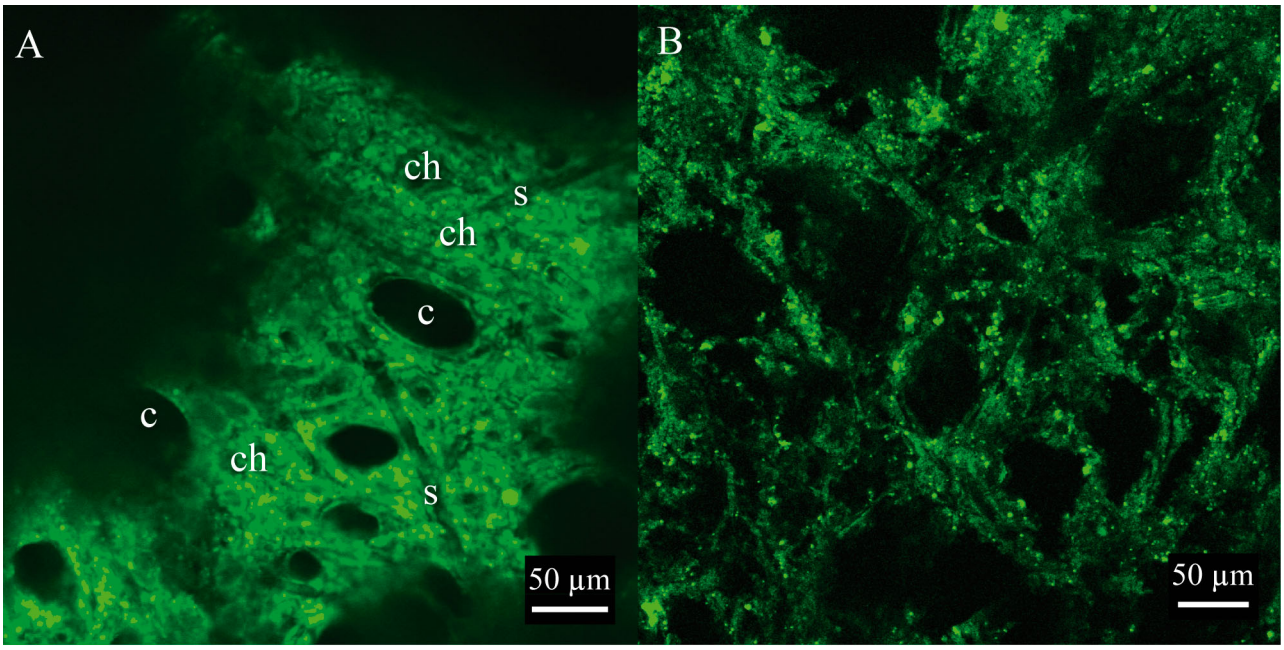



Fig. 8. Comparison of (A) temperatures ($T$, n = 6), (B) salinities (S, n = 6), (C) chlorophyll *a* concentrations (chl *a*, n = 8 and 9) and (D) weighted mean condition indices (CI, n = 6) of *Halichondria panicea* used in clearance experiments with high (i.e. during autumn) and low (i.e. during winter) CI. Central boxes show data between the 25$^{th}$ and the 75$^{th}$ percentiles, with the median represented as a line. The whiskers extend as far as the minimum and maximum values not considered as outliers. An outlier is represented by a circle and defined as a value lower than 1.5× the interquartile range (75$^{th}$ to 25$^{th}$ percentile). Significant differences (p < 0.05) are indicated by *



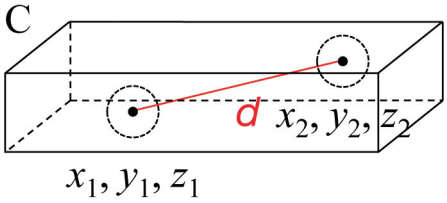




Fig. 9. Images from *z*-stacks of cryocut *Halichondria panicea* collected on (A) 2 December 2016 and (B) 1 May 2017 (both stained with green nanobeads), showing choanocyte chambers (ch), canals (c) and spicules (s). (C) Schematic drawing of *z*-stack (width: 450 µm, length: 450 µm, depth: 8–25 µm) containing 2 exemplary choanocyte chambers with the coordinates $x_1$, $y_1$ and $z_1$ and $x_2$, $y_2$ and $z_2$ and inter-chamber distance *d*

### 3.5. Density of choanocyte chambers

Sponges collected during winter (January 2017, low CI) and spring (May 2017, high CI) were analysed for distances between nearest neighbouring choanocyte chambers and volume-specific number of chambers (Fig. 9). In 14 samples taken during winter and 8 samples taken during spring, 98 and 45 inter-choanocyte chamber distances were identified, respectively. There was no difference in the mean distance between closest neighbouring chambers in winter (95.3 ± 76.5 µm, ± SD) and spring (86.1 ± 66.3 µm, Fig. 10A) (ANOVA, $F_{1,141}$ = 0.542, p = 0.463). Further, we observed a constant volume-specific number of choanocyte chambers (Fig. 10B) in winter (2429 ± 2117 chambers mm$^{-3}$) and spring (3235 ± 2348 chambers mm$^{-3}$, ANOVA, $F_{1,18}$ = 0.610, p = 0.445).

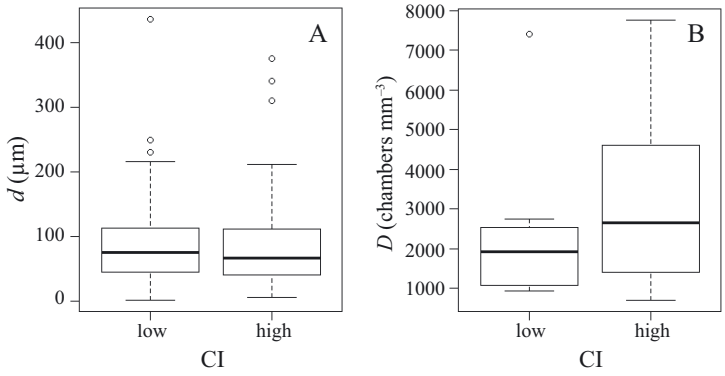



Fig. 10. (A) Distance between nearest neighbouring choanocyte chambers (*d*) and (B) choanocyte chamber density (*D*) of *Halichondria panicea* with low (i.e. during winter, about 0.7) and high (i.e. during spring, about 1.1) condition index (CI). Central boxes show data between the 25$^{th}$ and the 75$^{th}$ percentiles, with the median represented as a line. The whiskers extend as far as the minimum and maximum values not considered as outliers

## 4. DISCUSSION

The present study shows that the demosponge *Halichondria panicea* in temperate Danish waters exhibits a pronounced variation in CI during the year (Fig. 3D). Following a decrease during autumn and winter (November to February), the CI increased in early spring (February to May), followed by a rather stable CI during summer. CIs calculated for sponges from adjacent areas confirm the presented range,

Table 2. Volume-specific dry weight ($DW_v$) and ash-free dry weight ($AFDW_v$) calculated for a *Halichondria panicea* 'standard sponge' (sponge volume, $V_{sp}$ = 10 ml) based on DW and AFDW taken from the literature. n = number of sponges, CI: condition index (ratio of organic to inorganic matter) taken or re-calculated from the literature

| Month and year | Collection site | $V_{sp}$ (ml) | n | $DW_v$ (mg ml$^{-1}$) | $AFDW_v$ (mg ml$^{-1}$) | CI | Reference |
|---|---|---|---|---|---|---|---|
| March 1984 | Kiel Bight, Germany | 0.3–4 | 26 | | 32.0 | 0.55 | Barthel (1986) |
| March 1992 | Rosmø Sund, Denmark | 4–17 | 10 | 155.2 | 40.5 | 0.35 | Riisgård et al. (1993) |
| May 1994 | Kerteminde Fjord, Denmark | 1–80 | 20 | 70.0 | 24.0 | 0.52 | Thomassen & Riisgård (1995) |
| 9 March 2016 | Kerteminde Fjord, Denmark | 0.5–21.5 | 25 | 85.3 | 33.1 | 0.63 | This study, Table S1 |
| 26 May 2016 | Kerteminde Fjord, Denmark | 0.5–22.5 | 20 | 92.5 | 47.9 | 1.08 | This study, Table S1 |

even though they are slightly lower in March and May (Table 2). An intervening period with a marked fall and rise in CI during summer may be due to spawning (Barthel 1986). Changes in CI from spring to autumn may further be correlated with seasonal changes in temperature (Fig. 3A) and the concentration of bacteria and phytoplankton biomass (chl *a*; Fig. 3B). Depending on the conversion factor used for the carbon content of bacteria, results may differ by several orders of magnitude (Ferguson & Rublee 1976, Fenchel 1982, Fukuda et al. 1998). The low CI during winter, when the available particulate biomass (TCB) is minimal, reflects starvation of the sponge. However, the CI decreased with a time lag after TCB reached a minimum already in September. The phytoplankton species composition in specific months might differ among years. This could explain the difference in post-corrected PPCB, which would lead to variation in the TCB and affect the measured CI. Variation in CI implies variation in the organic content of the sponge (at constant content of inorganic components), i.e. number of living cells (due to lack of specialised energy storage cells in sponges; Barthel 1986).

The fact that the distance between neighbouring choanocyte chambers (Figs. 9 & 10) remains unchanged indicates that the filter-feeding apparatus is spared and thus ready for usage in early spring, when the food supply is again sufficient to cover the energetic demands of the sponge. For instance, seasonal changes in the partitioning of assimilated food energy into somatic growth and reproduction of the demosponge *Haliclona permollis* were studied by Elvin (1976), who found (over a period of 4 yr) that the somatic growth rates of intertidal and flat encrusting sponges on the Central Oregon Coast were minimal from December to April, reaching a maximum of about 1% d$^{-1}$ in the autumn, whereas the development of embryos was related to suspended food supply in late spring. Further, inorganic components of the sponge (i.e. spicules) may also alter with season (Schönberg & Barthel 1997), but only to a minor extent not significantly changing the DW:WW share over the seasons. This statement is supported by the measured volume-specific clearance rates in sponges with high (0.94 ± 0.17) and low (0.75 ± 0.10) CI, respectively (Fig. 7). In the hexactinellid deep-living sponge *Rhabdocalyptus dawsoni*, Leys & Lauzon (1998) found seasonal variation in sloughing of spicules that resulted in sponge size changes. This differs from *Halichondria panicea*, which appears unchanged during autumn and winter (Fig. 2). Finally, seasonality in spicule evolution may be different in sub-littoral hexactinellid sponges (*R. dawsoni*) and intertidal demosponges (*H. panicea*).

The present study supports previous findings by Barthel (1986), who described how the CI of *Halichondria panicea* varied over the season. Barthel (1988) suggested that mainly temperature and food availability controlled the CI; likewise, Koopmans & Wijffels (2008) argued that mainly temperature, algal biomass and POM controlled the annual variation in growth. From Fig. 3D, it appears that *H. panicea* has a low CI between November and February when the temperature was constantly low (Fig. 3A), and the available carbon in phytoplankton and free-living bacteria was low (Fig. 3C, Table 1). Based on the *F*/*R*-ratio, Riisgård et al. (2016) estimated the minimum maintenance requirements of *H. panicea* to be around 30 µg C l$^{-1}$. This estimation is in good agreement with the actual field data shown in Table 1. Thus in winter 2015/2016, the total biomass of 45.1 µg C l$^{-1}$ was just above this level, while in autumn and winter 2016/2017, the available biomass was below (TCB = 31.0 and 27.2 µg C l$^{-1}$, respectively), indicating starvation of the sponge in this period.

The carbon to chl *a* ratios (C:chl *a*) have recently been analysed for 7578 coastal seawater samples collected from Danish waters from 1990 to 2014 (Jakobsen & Markager 2016). The estimated 'grand arithmetic mean' for C:chl *a* ratios was found to be 41 ± 44 (mean ± SD). In the present work, we used a C:chl *a*

ratio of 40, which seems appropriate for the study site, but it should be noted that both seasonal and spatial dynamics affect the ratio. Jakobsen & Markager (2016) found that the C:chl *a* ratios were lowest during winter, about 15 across all stations. During spring, the ratio increased to summer values between 20 and 96, depending on the total nitrogen concentration. It appears from the contribution of free-living bacteria to the total suspended particulate biomass available to *H. panicea* that bacteria constitute a small but consistent part of the diet, around 17.8 ± 12.9% (Table 1), but that BCB remains below maintenance requirements year-round and therefore the sponge cannot survive solely on bacteria.

We found that bacterial concentrations varied during the year, from 0.3–1.2 × $10^6$ cells $ml^{-1}$, in agreement with earlier published values. For 2 sampling stations in the Skagerrak region and in the Baltic Proper, Rieck et al. (2015) reported bacterial concentrations of 0.52 ± 0.02 × $10^6$ and 0.74 ± 0.11 × $10^6$ cells $ml^{-1}$ in winter, respectively, and 0.52 ± 0.02 × $10^6$ and 1.13 ± 0.03 × $10^6$ cells $ml^{-1}$ in summer, respectively. Andersen & Sørensen (1986) presented bacteria concentrations from Limfjorden, Denmark, a highly eutrophic area, that ranged from 0.5 to 15.2 × $10^6$ cells $ml^{-1}$, with the lowest concentrations in autumn and winter (September to November 1983) and the highest bacterial concentrations in summer (June and July 1983). Further, Bernbom et al. (2011) showed log-transformed cell counts from various stations in Danish coastal waters with generally lower bacterial concentrations in winter (November 2009 and February 2010) than in spring and summer (April and June 2009), which confirms the seasonal variation presented herein. The pronounced difference between 'pre- and post-treated' phytoplankton biomass ($PPCB_{pre}$ and $PPCB_{post}$, Fig. 3C) may be related to possible size-dependent particle retention efficiencies, as reported by e.g. Ribes et al. (1999), and thus to possible exopinacoderm uptake of particles bigger than the ostia, as found in 2 Antarctic sponge species (Gaino et al. 1994). The present study has focussed on particulate organic carbon, but Yahel et al. (2003) suggested that DOC might also be a food source for sponges; however, Ribes et al. (1999) measured net production of DOC in feeding experiments, thus contradicting this suggestion. To our knowledge, no studies have shown that DOC is taken up by *H. panicea* (low microbial abundance sponge; Weisz et al. 2008), although DOC seems to play an important nutritional role in some (typically high microbial abundance sponge) species (Yahel et al. 2003, Mueller et al. 2014). Based on a review of the comprehensive literature, Jørgensen (1976, p. 316,318) concluded that it is 'mainly within restricted regions of the sea that dissolved organic compounds can be expected to be of importance as a source of energy to aquatic animals' and further that heterotrophic microorganisms may be 'far more important than the animals in metabolizing' DOC, which is in agreement with our present understanding of the microbial loop and the importance of heterotrophic bacteria that use DOC lost to the water by the primary producers (Fenchel 2008).

Our observation that seawater contains fewer free-living heterotrophic bacteria in winter (<$10^6$ bacteria $ml^{-1}$, Fig. 3B) is well documented (Pomeroy & Wiebe 2001) and is caused by the same factors (i.e. food limitation and grazing control by flagellates) as the lower bacterial concentrations in very oligotrophic waters (del Giorgio & Cole 1998). Free-living bacteria depend on the production of DOC, mainly due to photosynthesis of phytoplankton cells, which lose a substantial part of their photosynthates to the ambient water (Fenchel 2008). The concentration of bacteria is a function of available DOC in the water column, and the predation of various protozoans, especially flagellates, and therefore the bacterial density follows the primary production, and usually remains relatively constant around $10^6$ bacteria $ml^{-1}$ (Fenchel 2008).

When the temperature and available food (bacteria and phytoplankton) increased in March and April (Fig. 3B), the CI also increased (Fig. 3D), but it is difficult to separate the 2 factors as also stressed by Barthel (1986). A growth experiment with *H. panicea* conducted between April and August revealed a wet weight-specific growth rate of 0.6% $d^{-1}$ (Fig. 5), in good agreement with growth rate measured by Duckworth & Pomponi (2005) on *H. melanadocia* fed a mixed diet of bacteria and algal cells, whereas Thomassen & Riisgård (1995) who studied both *in situ* and laboratory growth of *H. panicea,* obtained specific growth rates of up to 4% $d^{-1}$. After the CI reached a maximum in summer (Fig. 3D), it first fell and subsequently recovered to an intermediate level until November, when water temperature and available food decreased. However, growth experiments by Duckworth & Pomponi (2005) and Thomassen & Riisgård (1995) indicate that growth and therefore CI are a function of food availability rather than temperature.

The clearance rates of *H. panicea* with either low or high CI (Figs. 7 & 8D) during autumn (1.5 ± 0.8 ml water (ml sponge)$^{-1}$ $min^{-1}$) and winter (1.9 ± 1.1 ml water (ml sponge)$^{-1}$ $min^{-1}$, mean ± SD) were nearly

identical, and in fair agreement with earlier laboratory studies (Riisgård et al. 1993: 2.7 ± 1.1 ml water (ml sponge)$^{-1}$ min$^{-1}$, Riisgård et al. 2016: 6.1 ml water (ml sponge)$^{-1}$ min$^{-1}$, Goldstein et al. (pers. comm.): 2.3 ml water (ml sponge)$^{-1}$ min$^{-1}$). Riisgård et al. (1993) also studied the effect of different temperatures on the clearance rate of *H. panicea* and found that an acute increase from 6 to 12°C caused a 4.3-fold increase in the mean clearance rate. Such a pronounced effect cannot be explained by temperature alone. Recently, Riisgård et al. (2016) showed that the filtration rate of *H. panicea* may vary considerably over time concurrently with often pronounced variations in the osculum size caused by disturbance when the seawater through-flow was stopped during filtration rate measurements. Acute changes of temperature in laboratory experiments are also likely to cause disturbance and thus pronounced variations in the filtration rate. However, no pronounced effect of temperature was observed in the present study, probably because the sponges were seasonally acclimated to the ambient water temperatures and studied under near optimal conditions.

The present observations of constant choanocyte chamber densities and mean distances between neighbouring chambers during winter and spring (Figs. 9 & 10), and likewise that the volume-specific clearance rate is near identical in low and high CI sponges, suggest that the density of filter-pump units is not affected by starvation. It remains unknown which components are primarily metabolised during starvation periods. The presented densities of choanocyte chambers (2429 ± 2117 and 3235 ± 2348 chambers mm$^{-3}$ in winter and spring, respectively) are low compared with 18 000 chambers mm$^{-3}$ reported by Reiswig (1975). As the CI decreased due to starvation, no reduction in the distance between adjacent choanocyte chambers was observed, which could have been the case if only cells other than choanocytes had been metabolised, not affecting the choanocyte chambers secured in position by inorganic components (i.e. spicules). Thus, a low CI seems to indicate an overall shrinkage (cf. Fig. 2, organic weight loss), whereby the relative contribution of inorganic components, i.e. spicules, increases. This is also supported by an insignificant trend towards lower DW:WW ratios during summer, when water makes up a greater part of the sponge biomass as shown by Schönberg & Barthel (1997). Clearly, sponges have evolved effectively to cope with starvation, and the present findings indicate a pronounced ability to withstand long periods of very low concentrations of suspended food particles.

*Acknowledgements*. This work was supported by a research grant (9278) from the VILLUM FONDEN (HUR). Thanks to Adrian G. López, Aikaterini Charitonidou, Ana Kovacic, Clémentine Prat, Thibault Neveu, Kasper Fjordside and Magnus Wahlberg for assistance during sponge collection and field work, to Per Andersen for help with phytoplankton data from the Great Belt, to Bernd Lüskow for creating the construction drawing, to Karl F. Lüskow for help with analysing the choanocyte chamber densities, to Rachael A. Kealy for improving the English style and to Josephine Goldstein, Lars Kumala, Niels T. Eriksen and 3 anonymous reviewers for constructive comments on the manuscript. We acknowledge the Danish Molecular Biomedical Imaging Center (DaMBIC, University of Southern Denmark) for the use of the bioimaging facilities.

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*Editorial responsibility: Pei-Yuan Qian, Kowloon, Hong Kong SAR*

*Submitted: January 29, 2018; Accepted: October 12, 2018*
*Proofs received from author(s): December 6, 2018*