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Measuring selectivity of feeding by estuarine copepods using image analysis combined with microscopic and Coulter counting

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Although estuarine zooplankters are generally believed to be detritivorous, high clearance rates by the estuarine copepods *Eurytemora affinis* and *Acartia tonsa* on natural estuarine microplankton have been reported in the literature. In order to enable detection of possible selectivity for these microplankton organisms over detritus, a method that measures clearance rates on total particulate matter is proposed. Image analysis is used to measure copepod gut contents, and combined with Coulter counter measurements of total particulate matter concentration, allows one to calculate clearance rates on the latter. The first results indicate that, in the Westerschelde estuary (SW Netherlands), both *E. affinis* and *A. tonsa* are capable of selecting dominant phytoplankton cells over the total particulate matter. The possibility of using image analysis as a sole technique in these studies is discussed.

Key words: *Acartia tonsa*, estuaries, *Eurytemora affinis*, feeding selectivity, image analysis.

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Introduction

The problem of measuring feeding on natural particulate matter, and hence selectivity of feeding by estuarine copepods

Evaluation of the impact of zooplankters on the carbon cycling of an ecosystem requires quantification of the predation pressure of the zooplankton communities on the primary producers and other biota, and parameterisation of these predation rates as a function of environmental conditions coupled with zooplankton production data. Estuaries and coastal areas form a specific study area in this context because, in these systems, potential prey organisms to the zooplankton (microplanktonic phyto- and zooplankters) occur in very low concentrations in the water, compared with the abundant detritus particles. Estuaries, in particular, are characterized by high particulate matter concentrations, with very complex composition. The bulk of the particulate mass in estuaries is formed by irregular aggregates or flocculates consisting mainly of detritus, with which phytoplankton and microbiota are associated (Eisma, 1991). An example of a microscopic view of a sedimented particulate matter sample from the brackish area of the Westerschelde estuary (SW Netherlands) is given in Figure 1.

Because of this dominance of detritus, estuarine zooplankters, such as the copepods *E. affinis* and *A. tonsa*, are generally believed to feed mainly on detritus (Heinle and Flemer, 1975; Heinle *et al.*, 1977; Hummel *et al.*, 1988). Yet considerable clearance rates by estuarine copepods on natural phytoplankton and microzooplankton, ranging up to 4 ml ind^{-1} h^{-1}, are reported in the literature (Robertson, 1983; Gifford and Dagg, 1988; Turner and Tester, 1989; Tackx *et al.*, 1995). These high predation rates on living phytoplankton suggest that, even in detritus-dominated environments, copepods are able to (occasionally) select specific food items. This suggestion is intriguing in relation to the selection capacities of copepods. Moreover, quantification of the degree to which estuarine zooplankton feeds on detritus, on the one hand, and living components on the other, is essential for adequate

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ecological and management-sustaining modelling of estuarine systems (Tackx *et al.*, 1995).

Clearance rates on estuarine phytoplankton and microzooplankton are usually measured from incubation experiments, using microscopic counting of the phytoplankton and microzooplankton species. To study selectivity, these clearance rates have to be compared with clearance rates on natural detritus or total particulate matter suspensions. This comparison is severely hampered because measurement of the latter is methodologically complicated by the above-described nature of the estuarine particulate matter. To arrive at realistic estimates of the natural predation rate, the degree to which the concentration of animals and incubation time can be increased in incubation experiments is limited (Roman and Rublee, 1980; Tackx and Polk, 1986). Microscopic counting of specific, clearly recognizable items such as phytoplankton species or zooplankters can be done with sufficient precision to obtain, within these experimental limitations, measurable differences in concentration of (some of) these organisms between grazing and control bottles from incubation experiments with estuarine copepods. On the contrary, the impact of the copepods on the high concentrations of the total particulate matter in these experiments is never important enough to create a difference in concentration between control and grazing bottles which is measurable by total particle counting devices, such as the Coulter counter or chemical measurements of particulate matter concentration, such as particulate organic carbon.

Alternative use of (natural) tracers in the copepod gut essentially focuses on components that are specific to certain living components, such as ^{14}C labelled phytoplankton, chlorophyll *a*, phaeopigments or vitamins (Haney, 1971; Mackas and Bohrer, 1976; Hapette and Poulet, 1990) and hence are not suitable for measuring predation rates on total particulate matter or detritus.

The problem of volumes and surfaces

Measurement of clearance rates on total particulate matter or detritus by the gut content approach requires that the concentration of these particles in the water, and their amount in the copepod gut, can be measured in equivalent terms of volume. Despite the difficulties encountered in incubation experiments, Coulter analysis provides a reproducible estimate of suspended particulate matter volume in estuarine samples. Variation coefficients on total volume concentration in the size range between 2.5 and 98 μm S.E.D. between four replicate Westerschelde samples are as low as 4% (unpublished results). A drawback of Coulter analysis is that it requires unpreserved samples (Klein Breteler, 1985), which restricts its use in the field to situations offering certain shipboard or laboratory facilities.

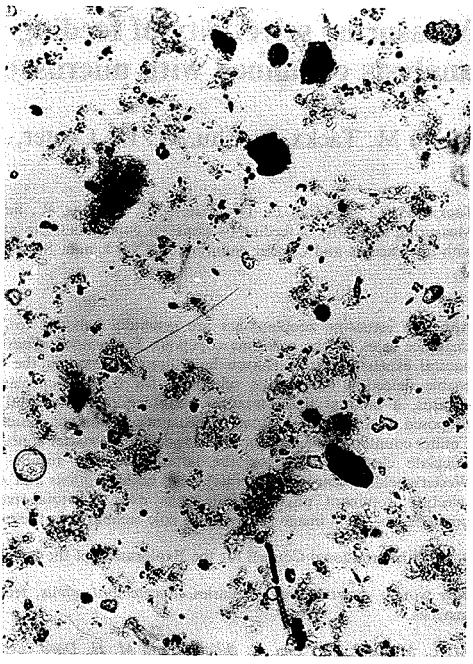

Figure 1. Example of a microscopic view of a sedimented particulate matter sample from the Westerschelde estuary.

Image analysis is recently providing a practical tool for accurate and fast analysis of preserved samples in marine ecology. It has been used for sizing, counting, and identification of zooplankton (Jeffries *et al.*, 1984), identification of algae (Balfoort *et al.*, 1992), and for determination of abundance, size, shape, volume, and surface area of marine organisms ranging from bacteria to fish larvae (Estep *et al.*, 1986; Verity *et al.*, 1992). Image analysis, being a uni- or two-dimensional technique, measures a number of object dimensions and/or the object area, from which data object volumes can be calculated using appropriate equations for the objects being measured. Formulae to calculate adequately volumes of the highly irregular shapes of natural estuarine particles are evidently impossible to develop. Yet, image analysis may provide a useful tool in specific estuarine situations. To evaluate these possibilities, the following aspects are considered:

Can natural particulate matter concentration be measured by image analysis?

Following Delesse's principle, the relative areas measured in microscopic transections of objects are

proportional to the relative volumes of these objects (Williams, 1980).

$$\frac{E(So)}{E(St)} \approx \frac{Vo}{Vt} \quad (1)$$

with So = sectional area of the component, St = sectional area of the containing volume, Vo = volume of component to be studied, Vt = containing volume, E = expected mean.

The volume of an object can be calculated from its sectional area if the sectional area and real volume of the containing volume and the thickness of the section are known. Hence, when the surface area of the particulate matter in a known volume of sample is quantified by image analysis, the volume of particulate matter can be calculated from the ratio of particulate matter area to cuvette area, if the cuvette's dimensions and the thickness of the layer in which the particulate matter has sedimented onto the bottom of the cuvette are known. This thickness being the height of the particles analysed, the problem is to assess the height of the bulk of the sedimented particles. This could be done if the real particle volume concentration was known (e.g. from Coulter analysis), and the height of the particles were constant for all the bulk particles in the sample. Height of microplanktonic organisms is very variable. However, in estuaries, microplankton contributes only a limited fraction of the total particulate matter and the bulk of particulate matter is formed by detritus-dominated aggregates (Fig. 1). These aggregates do not have a solid shape, but flatten out onto the bottom of the cuvette. Therefore, it is conceivable that a functional calculation of particle height, characteristic for sedimented particulate matter of a given estuarine situation, could be made from comparison of Coulter and image analysis data. This possibility was tested by comparing total particulate matter concentrations (measured as area) calculated from image analysis with total particulate matter concentrations measured by Coulter counter.

$$VPMc = h\,SPM \quad (2)$$

with VPMc = volume of particulate matter measured by Coulter counter (in $\mu m^3\ ml^{-1}$), h = average height of the particles (μm), and:

$$SPM = SPMi\,A$$

SPM = area of particulate matter calculated from image analysis ($\mu m^2\ ml^{-1}$), SPMi = fractional area of particulate matter measured in the image analysis ($\mu m^2\ view^{-1}$), A = conversion factor accounting for sample concentration and number of views $cuvette^{-1}$ at the magnification used.

Can image analysis measurements of gut content, with or without combination with Coulter data, be used to study the predation pressure of estuarine zooplankters on natural particulate matter, and possible selectivity of feeding for microplankton?

Measurements of the volume of particulate matter present in the gut of a copepod cannot be obtained directly, but have to be deduced from a two-dimensional observation of these guts. Considering the gut to be a sequence of cylinders with decreasing diameter (Penry and Frost, 1990), the volume can be calculated from microscopic measurements of the food parcel's width and length. Image analysis provides a tool for more rapid measurement of the food parcel's area than separate measurements of its dimensions, and a more accurate quantification of this surface in case the food parcel does not appear rectangular in the microscopic picture. For the food parcel's area to be an appropriate measurement of gut content, the area measured should be independent of the orientation of the animal in the analysis, consistent with the cylinder model of the gut. This was tested by comparing gut areas measured on the same individual, photographed in two different orientations.

Material and methods

Samples were taken at half depth in the brackish region of the Westerschelde estuary, at 10 ppt salinity. Zooplankton was caught with a 300 μm net. A fraction of the catch was stored in 4% formalin, the remainder placed in 10 l of natural water. Twelve bottles were filled with 1.4 l natural water and 4 × 50 adult *E. affinis* and *A. tonsa*, previously isolated under binocular microscope, were added to eight of these bottles. The remaining four bottles served as controls. From each bottle, a 400 ml sample was taken for Coulter analysis. Total particulate matter volume concentration in the size range 2.5–98 μm S.E.D. (VPMc) was measured using a combination of a 280 and 100 μm tube. Copepods removed from the samples taken from the grazing bottles were counted so that they could be corrected for in the calculation of clearance rates. After Coulter analysis, the remaining samples were reduced to 250 ml and fixed with lugol's solution for phytoplankton counts. The control and grazing bottles were stored in an incubator and an outboard pump was used to maintain environmental temperature. The bottles were regularly rotated to prevent settling of the particulate matter. After 14 h, 250 ml samples were taken from each bottle and fixed with lugol's solution.

In the laboratory, the samples were concentrated to 60 ml and the dominant phytoplankton species counted on 5 ml subsamples under an inverted microscope at 10 × 20 and 10 × 40 magnification. For those phytoplankton species in which significant differences in the original counts could be detected between control and grazing bottles (Mann-Whitney, $p < 0.05$), clearance

rates (Fph) were obtained from the calculated natural concentration of these species following Frost (1972).

From the same samples, slides were taken under the inverted microscope at 10×40 magnification for determination of particulate matter concentration by image analysis; 20–25 copepods from the samples which had been fixed with formalin on board were isolated, slightly bleached with HClO and photographed individually under inverted microscope at a 10×10 magnification.

In view of the fact that the aim was to detect selectivity for phytoplankton, care was taken to develop the image analysis procedure in such a way that clearance rate values on total particulate matter would be rather over- than under-estimated.

Total particulate matter area was quantified from the slides using a IBAS II Kontron image analyzer. The images were imported from the microscopic slides with a black and white camera. After different contrast and contour enhancement manipulations on the real image, the areas to be measured were discriminated by their respective grey values. While setting the grey level thresholds, care was taken not to overestimate the surface of the particulate matter. SPMi values were measured on the digitized image. Calibration for calculation of the factor A was done using a calibration graticule.

On the copepod slides, surface of the food parcels (called gut area in the following) was quantified by a specific software program. Gut area was quantified by grey shading, as for particulate matter, this time with an overestimation rather than an underestimation. Gut areas were measured on the same animal in two different positions to verify that gut area is independent of the animal's orientation in the analysis. Gut area was calculated in μm^2, following calibration.

Width of the food parcels in the copepod guts was measured on the copepod slides under binocular microscope. These widths were generally equal to the gut width at the place where the food parcel was located. The length of each food parcel was calculated by considering its surface to be rectangular, dividing the gut area by the average gut width. The volume of each food parcel was calculated assuming the food parcel to be a cylinder with a diameter equal to the average parcel width. The gut content (G, in μm^3 ind^{-1}) was calculated as the sum of volumes of all food parcels in the gut.

Clearance rates on total particulate matter (FT) were calculated as:

$$FT = \frac{G}{GPT \times VPM} \quad ml\ ind^{-1}\ h^{-1} \qquad (3)$$

with GPT = gut passage time, chosen as a minimum of 10 min, VPM = particulate matter volume concentration calculated from image analysis as h SPM (μm^3 ml^{-1}).

Results

The h values calculated from Equation (2) varied between 2.12 and 4.25 μm. The average was 2.69 ± 0.71 μm ($n = 8$).

Figure 2A, B shows the two replicate gut area measurements on the same copepod in two orientations for *E. affinis* and *A. tonsa* respectively. In most cases the animals were analysed in two different lateral positions, because of difficulty keeping them in the dorso-ventral position while taking the picture. For both *E. affinis* and *A. tonsa*, gut content was insignificantly different between both replicates (Wilcoxon signed rank test, $p > 0.05$). A tendency for lateral analysis to give higher area values was noted, however.

The measurements performed on food parcel width showed that anterior food parcels tended to be wider than posterior food parcels, but the means did not differ significantly (Mann Whitney, $p > 0.05$). Therefore, a global average width of 36.13 ± 9.94 μm for *E. affinis* and 36.75 ± 11.37 μm for *A. tonsa* was considered.

From the microscopic counts of the samples from the incubation experiment eight phytoplankton species were found to be present in considerable concentrations (>5 cells ml^{-1}). The centric diatom *Coscinodiscus commutatus* formed a dominating volume peak (0.3 ppm), and was the only species for which a significant difference in concentration between control bottles and grazing bottles was detected. Clearance rates calculated on this species varied between 0.79 and 3.64 ml ind^{-1} h^{-1} for *E. affinis* and 0.96 and 1.54 ml ind^{-1} h^{-1} for *A. tonsa*.

FT values calculated following Equation (3) are shown in Figure 3A, B. They varied between 0.04 and 0.36 ml ind^{-1} h^{-1} for *E. affinis* and between 0.02 and 0.28 ml ind^{-1} h^{-1} for *A. tonsa*.

This figure also shows that for both *E. affinis* and *A. tonsa* FT values were an order of magnitude lower than Fph values measured on *C. commutatus*.

Discussion

Can particulate matter concentration be measured by image analysis?

The estimated h values of the particles are fairly constant, between 2.12 and 4.25 μm. This would correspond to the S.E.D. of the smallest particles measured in the Coulter size distribution. It is known in natural particulate matter samples that the small particles are the most abundant (Sheldon *et al.*, 1972), and this is certainly the case in estuarine waters (Chervin *et al.*, 1981; Castel and Feurtet, 1986; Irigoien and Castel, 1994). As can be seen from Figure 1, most of the area in the sedimented samples is made up of aggregates, and these aggregates are mainly composed of small particles. An average "particle height" of 2.69 μm does not therefore seem unrealistic. It should also be kept in

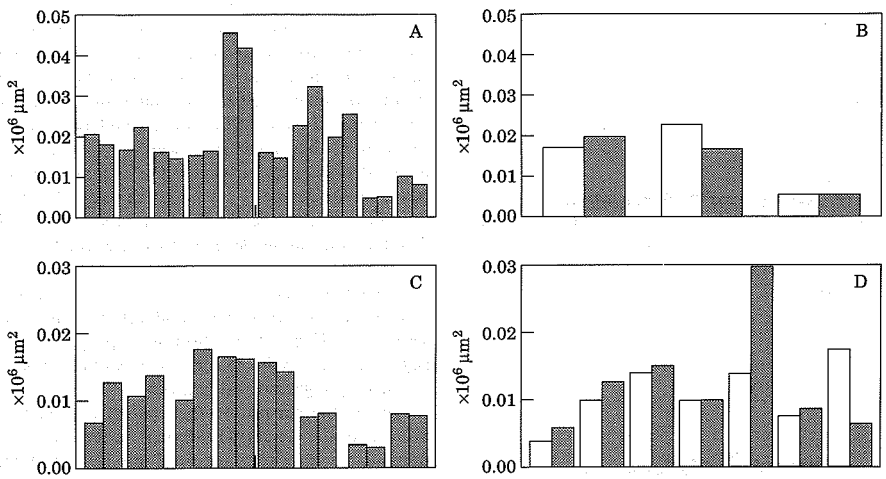

Figure 2. Gut area measured on one individual copepod in pictures taken in two lateral (▨) positions or in lateral (▨) and dorso-ventral (□) positions (A, B) *E. affinis*; (C, D) *A. tonsa*.

mind that the combination of VPM and SPM measurements does not necessarily result in the exact true dimension of the particles studied, but is basically a conversion factor between Coulter and image analysis results. A bias in the estimation of h could occur when particles are superimposed upon each other in the cuvette. In our analysis, sample size was adjusted in order to obtain a good spread of the particulate matter over the area of the cuvette (cf. Fig. 1). Moreover, any such overlap would have added to the strategy of underestimating total particulate matter concentrations from SPMi values. Should further studies on estuarine particulate matter show that conversions between VPM and SPM measurements are fairly constant in certain situations (e.g. areas, seasons, tidal phases), image analysis data on particulate matter area could be used to estimate particulate matter volume concentrations, using a limited number of standardization trials combining image analysis and Coulter data.

Can image analysis measurements of gut content, with or without combination with Coulter data, be used to study the predation pressure of estuarine zooplankters on natural particulate matter, and possible selectivity of feeding for microplankton?

The duplicate measurements of gut area on individual copepods show that the results obtained are independent of the position of the copepod during the analysis. Nevertheless, it can be seen from Figure 3 that large deviations between duplicate observations sometimes occur, necessitating careful judgement by the operator and numerous observations. The rather large variability in gut content observed between individuals corresponds with reports by Kleppel *et al.* (1988) on gut content and cinematographic observations of significant animal to animal variability in calanoid feeding behaviour (Turner *et al.*, 1993). The tendency for anterior food parcels to be wider than posterior ones could be explained by the fact that anterior measurements comprised not only food parcels in the anterior mid-gut, but also some which were probably not yet surrounded by the peritrophic membrane.

The fact that clearance rates calculated from G and VPM values are an order of magnitude lower than those measured on *C. commutatus* indicates that the latter was selected by both copepod species studied. Clearance rates measured in similar incubation experiments using microscopic counting have been shown to be realistic, underestimating rather than overestimating predation pressure on naturally occurring phytoplankton in the Westerschelde (Tackx *et al.*, 1995). As stated in the material and methods section, gut content measurements by image analysis were purposely over- rather than under-estimated, and SPM values conversely. Also, the estimation of 10 min for gut passage time is surely a minimum, as gut passage times measured for these species in estuaries vary between 15 and 40 min (Irigoien *et al.*, 1993; Tackx *et al.*, 1995). One possible source of underestimation is loss of gut content by defecation or regurgitation, as copepods in our experiment were not narcotized prior to fixation. Most of the guts observed

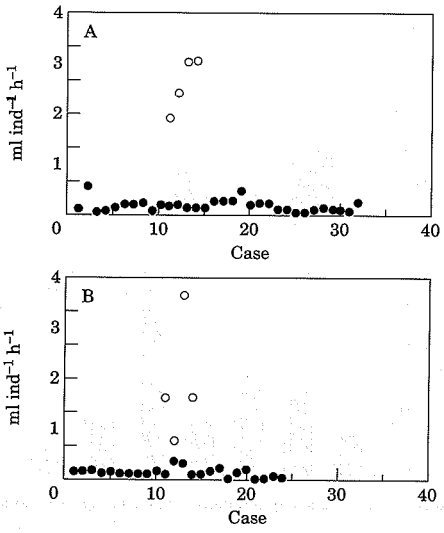

Figure 3. Fph values (○) and FT values (●) measured for (A) *E. affinis* and (B) *A. tonsa*. Fph values are plotted at random positions on the x axis, which represents the cases (= animals analysed for gut content). See text for further explanation.

were filled to between 20 and 40%. This is a little lower than the gut fullness of 40% reported by Kleppel *et al.* (1988), who worked with narcotized animals. In view of the large difference between Fph and FT values, and the above explained overestimation of FT values, even a three-fold underestimation of gut content would not suffice to contradict the indication of phytoplankton selection. The contribution of inorganic particles to the total volume of suspended particulate matter in the Westerschelde is small (Wartel, pers. comm.), so the method used indeed measures selection between phytoplankton and the remainder of the suspended organic particles in the 2.5–98 μm size range. Evidently, the present example allows one merely to demonstrate the possibilities of the approach, and needs further experiments to complement it. Nevertheless, the magnitude of the difference between Fph and FT values measured in our experiment suggests that, even though the proposed techniques are rather imprecise, they may be functional in the specific context of studying selectivity of estuarine zooplankters feeding.

Any ecologically important selectivity for microplankton should be reflected in considerably higher Fph than FT values. Under these conditions, and because of the high particulate matter concentrations in estuaries, the variables used to calculate ingestion rates on total particulate matter do not need to be measured with high precision in order for a significant difference to be detected between Fph and FT values. In the example presented here, using SPM values instead of VPM values for the calculation of FT still resulted in significant differences between Fph and FT values (Mann Whitney, $p < 0.01$). Assuming that h values can be considered as 1, SPM values could be used in the calculation, allowing measurement of FT values on fixed material only. Arguments for this assumption can be based on the nature of the estuarine particulate matter, the low h values calculated in our experiment, and the fact that this would lead to an overestimation of FT values.

Using an overestimation approach for FT calculation provides a conservative method for detecting feeding selectivity. This conservatism is appropriate, as the suggestion of selective feeding by estuarine zooplankters is generally regarded with criticism.

Finally, the perspective that typical high concentrations and the aggregate-forming nature of estuarine particulate matter may be turned into a methodological advantage rather than a disadvantage, provides an impetus for looking further into the potential of this approach.

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