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HPLC analysis of algal pigments to define diet of sea urchins

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A method for a qualitative analysis of sea urchin diet is based on the characterization of the photosynthetic pigment indices of the major algal groups in the sea urchin gut. The pigments were separated by reverse-phase high performance liquid chromatography (RP-HPLC). This study demonstrated that HPLC is a better method to estimate chlorophyll-*a* in the gut contents than the conventional spectrophotometric methods which overestimate the amounts by including chlorophyll-*a* breakdown products. Three sea urchins species, *Paracentrotus lividus*, *Psammechinus miliaris* and *Sphaerechinus granularis*, settled on the loose-lying coralline algae (maerl) in the Bay of Brest (France), were used in this study. The algal pigments identified within the gut contents included chlorophylls-*a*, -*b*, -*c*, fucoxanthin, lutein, β,ε-carotene and β,β-carotene. The presence of chlorophylls and carotenoid biomarkers was used to characterize the three algal groups: Rhodophyceae, Chlorophyceae, Phaeophyceae in estimating sea urchin diet. The pigment analysis reported here demonstrated that the three species of sea urchins investigated mainly consumed Rhodophyceae which dominate the epibenthic flora in the study area.

INTRODUCTION

Sea urchin diets are difficult to analyse and quantify because of taxonomic problems in identifying algal fragments and because microscopical examination is time-consuming. Yet, dietary information is needed for studying the role of these herbivores on the algal communities as well as for management of urchin fishery. A method based on the characterization of the photosynthetic pigment indices as diets using high performance liquid chromatography (HPLC) is described for analysing the algal groups in the sea urchin gut contents. Although pigments may vary among cells within a taxon or between taxa, the presence of biomarker pigments generally reflects the composition of the respective algal groups. The method had been first used on marine filter-feeding organisms (Hawkins et al., 1986; Buffan-Dubau et al., 1996). We applied this method for the first time to macrograzers.

MATERIALS AND METHODS

Five to ten specimens of *Paracentrotus lividus* (Lamarck), *Psammechinus miliaris* (Gmelin) and *Sphaerechinus granularis* (Lamarck), were collected monthly from the Bay of Brest (Brittany, France) from coralline algae beds which constitute a favourable substratum to macrophyte development. The algae were mostly Rhodophyceae as indicated by a dominant biomass of red algae (>80%) dominated by *Cryptopleura ramosa* (Solander & Turner) and *Microcladia glandulosa* (Hudson) and the following species ratios Rhodophyceae/Phaeophyceae=11:1 and Rhodophyceae/Chlorophyceae=9:1. Urchins were collected by SCUBA diving between 1000 and 1200 hours from February–June 1998 for *P. miliaris* and *S. granularis* and February–August for *Paracentrotus lividus*. To avoid the influence of urchin size on nutrition, a narrow urchin size range was used: 33 mm (ambitus without spines) for *Psammechinus miliaris*, 51 mm for *Paracentrotus lividus*, and 105 mm for *S. granularis*. The gut contents were carefully separated from the gut wall, immediately frozen and stored in the dark at −20°C until further analysis. In the following month, three replicates of each individual gut contents was extracted by grinding in cold 90% acetone (20 ml), then incubated in the dark 2 h at 4°C. Debris were separated from the pigments by centrifugation at 4°C for 15 min at 5000*g*. The pigments were analysed by spectrophotometric and HPLC methods and the results compared. Chlorophyll-*a*, -*b* and -*c* concentrations in 90%-acetone extracts were determined by spectrophotometry using the equations of Jeffrey & Humphrey (1975), whereas the acid-spectrophotometric method of Lorenzen (1967) was used for measuring chlorophyll-*a* and phaeopigment concentrations. The method described by Wright et al. (1991) was modified for the analysis of chlorophylls, chlorophyll-degradation products and carotenoids by HPLC to allow the delivery of a binary solvent system. Aliquots of pigment extracts (50 μl) were directly injected into a Waters Associated HPLC system (Waters, Milford, USA) equipped with reverse-phase column C18: Spherisorb® S5ODS2 (Waters, Milford, USA). Solvent A consisted of 0.3 M ammonium acetate in methanol, water and acetonitrile (51:13:36, v/v/v) and solvent B of acetonitrile and ethyl acetate (30:70, v/v). All pigments detected with either a Waters 486 Tunable Absorbance Detector set at 436 nm or a Waters 474 Scanning Fluorescence Detector set at excitation and emission wavelengths of 434 and 670 nm respectively were eluted within about 30 min using a linear gradient from 100% A to 100% B at a flow rate of 0.8 ml min^{-1} (Figure 1). Calibration methods for HPLC were applied as indicated by Mantoura & Repeta (1997). Before its application to the gut contents, this

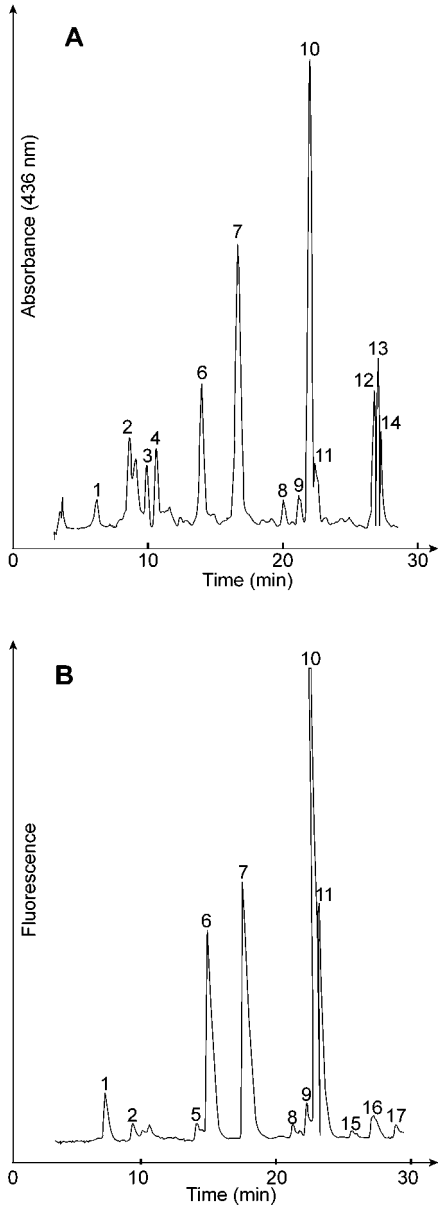

Figure 1. Chromatograms of gut contents extracts from *Paracentrotus lividus* collected in February 1998. (A) Absorbance chromatogram. (B) Fluorescence chromatogram. The numbered peaks are identified as follows: 1, chlorophyllide *a*; 2, chlorophyll-*c*; 3, 19′ butanoyloxyfucoxanthin; 4, fucoxanthin; 5, phaeophorbide *a*; 6, violaxanthin; 7, lutein+zeaxanthin; 8, chlorophyll-*b*; 9, chlorophyll-*a* allomer; 10, chlorophyll-*a*; 11, chlorophyll-*a* epimer; 12, *β*,*ε*-carotene; 13, *β*,*β*-carotene; 14, cis-*β*,*ε*-carotene; 15, phaeophytin *b*; 16, phaeophytin *a*; 17, pyrophaeophytin *a*.

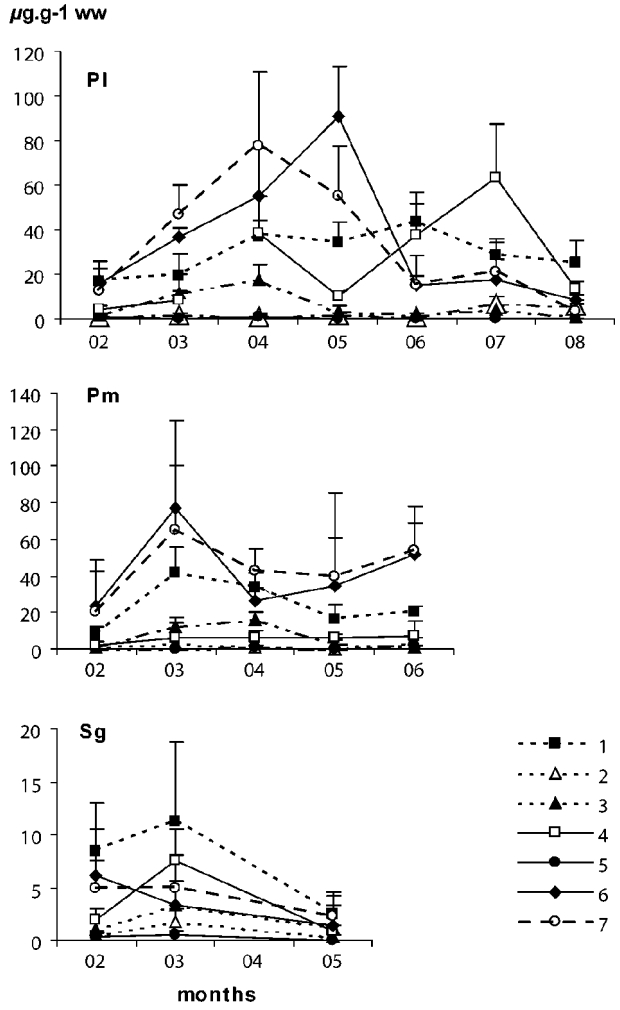

Figure 2. Amount of selected pigments biomarkers in gut contents of the three urchins sampled in the Bay of Brest (mean ±SE) (PI, *Paracentrotus lividus;* Pm, *Psammechinus miliaris;* Sg, *Sphaerechinus granularis*). 1, Chlorophyll-*a*; 2, chlorophyll-*b*; 3, fucoxanthin; 4, lutein+zeaxanthin; 5, violaxanthin; 6, *β*,*ε*-carotene, 7, *β*,*β*-carotene.

method had been successfully tested on different algal species representative of the three main classes, i.e. Chlorophyceae, Rhodophyceae and Phaeophyceae.

RESULTS AND DISCUSSION

The mean concentration of chlorophyll-*a* in the gut contents of *Paracentrotus lividus*, *Psammechinus miliaris* and *Sphaerechinus granularis* in February was 59.6 ±17.4; 37.8 ±23.6 and 26.5 ±7.12 $\mu g\,g^{-1}$ ww respectively when determined by the spectrophotometric methods of Jeffrey & Humphrey (1975), 48.8 ±14.8; 30.2 ±17.9 and 21.3 ±6.5 $\mu g\,g^{-1}$ ww when measured by the method of Lorenzen (1967) and 16.6 ±9; 7.8 ±4.7 and 8.5 ±4.5 $\mu g\ g^{-1}$ ww when HPLC was used. The concentrations determined by the spectrophotometric methods were significantly higher than those obtained by HPLC ($P<0.05$, one-way analysis of variance).

Figure 1A illustrates a typical HPLC absorbance chromatogram and shows the elution pattern of a range of chlorophyll and carotenoid pigments detected in *Paracentrotus lividus* gut contents sampled in February. In addition to chlorophyll-*a*, the major pigments characterized from algae including chlorophyll-*b*, chlorophyll-*c*, fucoxanthin, lutein, zeaxanthin, *β*,*ε*-carotene, *β*,*β*-carotene were identified. However using this HPLC method, lutein and zeaxanthin were coeluated. A typical fluorescence chromatogram of the HPLC-separated pigments from *P. lividus* (Figure 1B) highlights the numerous chlorophyll-*a* breakdown products and derivatives of various polarity indicative of feeding and digestive processes. They consisted of chlorophyllide-*a*, phaeophorbide *a* and phaeophorbide *a*-like, allomer and epimer

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of chlorophyll-*a*, phaeophytin *a* and pyrophaeophytin *a*. Moreover, in February samples, (chlorophyll-*a*+phaeophytin *a*) levels ranged within 2.4 and 11.4 μg g^{-1} ww by species with phaeophytin *a* accounting for 22 to 50% of the overall (chlorophyll-*a*+phaeophytin *a*) content. It is likely that the pigment breakdown products were partly responsible for the differences observed in chlorophyll-*a* concentrations according to the method used. Indeed, as their absorption properties are similar to those of chlorophyll-*a*, they were likely quantified as chlorophyll-*a* by the spectrophotometric methods. Although the acid-spectrophotometric method of Lorenzen (1967) allowed us to refine chlorophyll-*a* concentrations by integrating in calculations a correction factor representative of phaeopigments content, the results provided by this technique did not differ significantly from the ones given by the spectrophotometric method of Jeffrey & Humphrey (1975). Noticeable fluctuations in the concentrations of the marker pigments were detected in the gut contents of the sea urchins (Figure 2). Chlorophyll-*a*, lutein+zeaxanthin and β-carotene were the most abundant pigments. However pigment levels were much lower in *S. granularis* than in the two other species. Violaxanthin remained at a low level in the three sea urchin gut contents. During the studied period, the analysis of gut pigment composition demonstrated the predominance of β-carotene and lutein+-zeaxanthin along with the reduced amount of chlorophyll-*b*. Lutein is indicative of Rhodophyceae and Chlorophyceae. But, if we assume that chlorophyll-*b* dominates in Chlorophyceae since a chlorophyll-*b*-to-lutein ratio between 1.31 and 1.55 is typical of Chlorophyceae like *Cladophora glomerata* and *Enteromorpha intestinalis* (Bianchi et al., 1997), then the dominance of lutein+zeaxanthin in the gut contents will be due to the presence of Rhodophyceae.

β,ε-carotene and, β,β-carotene were not totally separated (Figure 1) and their quantification would not be fully accurate. However β,ε-carotene is usually lower in Chlorophyceae than in Rhodophyceae as indicated by the analysis of the different algae tested in the prelude of this study and the results of Bianchi et al. (1997). The presence of this pigment in the gut sea urchin can thus be used as a biomarker of Rhodophyceae. The observations reported here showing the presence of these two pigments with a low level of chlorophyll-*b* suggest that the *in situ* diet of the three species was mainly based on Rhodophyceae. Nevertheless, in March and April, the level of fucoxanthin in the gut samples of *P. lividus* and *Psammechinus miliaris* indicated a temporarily high contribution of Phaeophyceae to their diet despite a Rhodophyceae to Phaeophyceae ratio of 30:4 in the environment at this period. Then the fucoxanthin level stayed low while the brown algae diversity was increasing in the field. The season- and species-dependent character of the sea urchin diet should be confirmed by a further analysis.

REFERENCES

Bianchi, T.S., Kautsky, L. & Argyrou, M., 1997. Dominant chlorophylls and carotenoids in macroalgae of the Baltic Sea (Baltic proper): their use as potential biomarkers. *Sarsia*, **82**, 55–62.

Buffan-Dubau, E., De Wit, R. & Castel, J., 1996. Feeding selectivity of the Harpacticoid copepod *Canuella perplexa* in benthic muddy environments demonstrated by HPLC analyses of chlorin and carotenoid pigments. *Marine Ecology Progress Series*, **137**, 71–82.

Hawkins, A.J.S., Bayne, B.L., Mantoura, R.F.C. & Llewellyn, C.A., 1986. Chlorophyll degradation and absorption throughout the digestive system of the blue mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology*, **96**, 213–223.

Jeffrey, S.W. & Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochemie und Physiologie der Pflanzen*, **167**, 191–194.

Lorenzen, C.J., 1967. Determination of chlorophyll and pheopigments: spectro-photometric equations. *Limnology and Oceanography*, **12**, 343–346.

Mantoura, R.F.C. & Repeta, D.J., 1997. Calibration methods for HPLC. In *Phytoplankton pigments in oceanography: guidelines to modern methods* (ed. S.W. Jeffrey et al.), pp. 239–260. Paris: UNESCO.

Wright, S.W., Jeffrey, S.W., Mantoura, R.F.C., Llewellyn, C.A., Bjornland, T., Repeta D.J. & Welschmeyer, N., 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine Ecology Progress Series*, **77**, 183–196.

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