Cah. Biol. Mar. (1996) 37 : 33-48

Eulalia viridis (Polychaeta: Phyllodocidae) is a complex of two species in northern Europe: Results from biochemical and morphological analyses

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Abstract: Given that relatively simple electrophoretic methods have repeatedly proven their usefulness in sorting out difficult species complexes, it seemed a promising approach to apply them to various local populations of *E. viridis* in order to test whether this species name is in fact being used for more than a single species.

Specimens were collected from geographically separated local populations in the Channel region (Saint-Efflam, Granville) and the North Sea (Newcastle, Norderney, Helgoland, Helsingør and Tjärnö). Particular enzymes and general protein patterns of individual animals were compared by means of isoelectric focusing (IEF), and the animals were examined for morphological differences. Distinct differences between the IEF band patterns of specimens from the western and eastern sites were indeed observed, and these were found to be correlated with differences in the morphology and size of the dorsal cirri and the size of the proboscideal papillae. Therefore, it became necessary to subdivide the material into two species, *E. viridis* for that found in Sweden, Denmark and Germany and *E. clavigera* for that from France and England.

Résumé : Des méthodes électrophorétiques relativement simples se sont révélées utiles pour séparer des complexes d'espèces difficiles et il a paru intéressant de les appliquer à différentes populations d'*Eulalia viridis* dans le but d'examiner si sous ce même nom il y aurait en fait plus d'une espèce.

Les spécimens ont été récoltés dans des populations géographiquement séparées sur les côtes de la Manche (France : Saint-Efflam et Granville) et de la Mer du Nord (Newcastle, Norderney, Helgoland, Helsingør and Tjärnö). Les profils d'enzymes particulières et de protéines générales d'individus isolés ont été comparés au moyen de l'isoélectrofocalisation (IEF) et les différences morphologiques des animaux ont été étudiées. Différents profils obtenus par isoélectrofocalisation, entre des spécimens des sites ouest et est, ont été observés et sont corrélés à des différences dans la morphologie et la taille des cirres dorsaux, et la taille des papilles de la trompe. De ce fait il devient nécessaire de distinguer deux espèces, *E. viridis* pour les spécimens récoltés en Suède, Danemark et Allemagne et *E. clavigera* pour ceux de France et d'Angleterre.

Keywords: *Eulalia viridis* complex, isoenzymes, general protein, morphological data, Phyllodocidae, Polychaeta.

Introduction

The Phyllodocidae Ørsted, 1843 is a large family of benthic polychaetes containing about 350 species. Papers dealing with the systematics of the Phyllodocidae (Bergström 1914, Ushakov 1972, Pleijel 1991, Eibye-Jacobsen 1993) have, however, pointed out that the taxonomic status of many species is still uncertain.

Eulalia viridis (Linnaeus, 1767) is one of these problematic taxa. This species is common in intertidal and

Reçu le 1er novembre 1995 ; accepté après révision le 2 mai 1996
Received 1st November 1996; accepted in revised form 2 May 1996.

subtidal coastal areas at depths less than 50 m (Ushakov 1972), and it has been reported from throughout the northern hemisphere (Fauvel 1923, Pettibone 1963, Pleijel 1991, Eibye-Jacobsen 1993). *E. viridis* usually lives on firm substrates, such as rocks, barnacle aggregates, calcareous tubes of serpulid polychaetes and bivalve shells. Local populations along the coasts of Northern Europe differ in colouration and time of reproduction; on the western coast of Sweden the reproductive cycle starts 4 to 6 weeks earlier than on the coast of the United Kingdom and France (Olive 1975, Pleijel 1993), which has led to the hypothesis that *Eulalia viridis* may represent a species complex.

The aim of this study was to use IEF (isoelectric focusing method) to survey individuals of geographically distinct sampling stations of *E. viridis* to test the hypothesis that different species exist along the coasts of northern Europe and to reexamine the morphology of the animals from these areas, if any differentiation is established.

Materials and methods

A total of 180 phyllodocid individuals were collected from tidal and subtidal stations between May and September of 1994. The location and the depth of collection sites are shown in Table 1 and Figure 1. *Eulalia* specimens were sampled by hand from shores in France ("Roche Rouge" at Saint-Efflam, Brittany and Granville, Normandy) and in the United Kingdom (Newcastle), and transported alive to Osnabrück in coolers. Other individuals were sampled by diving off the coast of Tjärnö (western Sweden). Aggregates of *Pomatoceros* tubes, which are also inhabited by *Eulalia*, were brought to the laboratory and kept in plastic tubes filled with seawater. As the oxygen content decreased, the phyllodocids left the serpulid tubes and could be easily collected with a pipette from the water surface. Samples of *Laminaria hyperborea*, obtained by diving from Helgoland, were treated similarly. One additional specimen was collected by the research vessel "Ophelia" (Marine Biological Institute, Helsingør, Denmark), and another was collected by hand within aggregates of *Balanus* on the island of Norderney, Germany.

The polychaetes were transferred individually into clean seawater, freed of detritus and anaesthetized with $MgCl_2$ (7.5%). The length of the body was measured and segments were counted. Thereafter, the proboscis with the anterior

Table 1: Phyllodocid specimens examined from different European tidal and subtidal stations.
Tableau 1 : Spécimens de phyllodocidés examinés dans différents sites du littoral et de l'infralittoral des côtes d'Europe.

No.	Date	Station	Depth (m)	Proposed species names*	Sample size
1.	21.06.1994	"Roche Rouge"at Saint-Efflam (Brittany), in beds of *Mytilus edulis*	Eulittoral	*Eulalia clavigera**	46
2.	22.07.1994	Newcastle upon Tyne (England), 4 km north of the mouth of the river Tyne in the southern part of Cullercoats Bay, in rock crevices	Eulittoral	*Eulalia clavigera**	36
3.	26.07.1994	Granville (Normandy), in green algae and blocks of *Balanus* on rocky substrate	Eulittoral	*Eulalia clavigera**	31
4.	18.09.1994	Norderney, in aggregates of *Balanus*	Eulittoral	*Eulalia viridis**	1
5.	01.05.1994-09.05.1994	Helsingør (Denmark), Øresund, in tube of *Pomatoceros triqueter* on *Modiolus modiolus*	20	*Eulalia viridis**	1
6.	24.08.1994-02.09.1994	Tjärnö (Sweden) with *Pomatoceros triqueter* tubes at Klinken	7	*Eulalia bilineata*, *Eulalia viridis**, *Phyllodoce maculata*	1 58 5
7.	26.09.1994-29.09.1994	Helgoland/Nordost-felswatt/in holdfasts of *Laminaria hyperborea*	7	*Eulalia viridis**	1

Figure 1: Sampling stations and corresponding proposed species names: *Eulalia clavigera* (squares) and *Eulalia viridis* (dots). Numbers: see table 1.

Figure 1 : Sites d'échantillonnages et noms d'espèces proposés : *Eulalia clavigera* (carrés) et *Eulalia viridis* (ronds). Chiffres : voir tableau 1.

10-20 segments, several segments of the middle part of the body and the pygidium with the posterior 10-20 segments were removed and fixed in formalin (4%) for morphological analyses. The remaining parts of the worm were separated into portions of equal size, rinsed in distilled water to remove intestinal contents and salts and individually frozen at -70°C in 1.5-ml Eppendorf tubes.

Tissues were prepared for IEF (isoelectric focusing) by homogenizing the portions in buffer (0.05 M TRIS/HCl, pH 7.5) using an electric drill at 3000 rpm for several seconds. IEF was performed in 0.5-mm thick polyacrylamide gels (T = 7.5%, C = 3%) onto a horizontal system (LKB Multiphor II). The gel solution contained 7% of carrier ampholyte preparation, exclusively limited to the range pH 4-9 for isozymes, and a mixture of ampholyte preparations covering the range pH 4-7 (60%) and pH 4-9 (40%) for general protein patterns. The gels were cooled down to 10°C during focusing. The electrode wicks were located 9 cm and 15 cm apart for isozymes and the general protein studies, respectively. The electrode solutions contained 0.025 M aspartic acid and 0.025 M glutamic acid for the anode and 0.025 M arginine, 0.025 M lysine and 2 M ethylene diamine for the cathode. Application foils with a slot width of 7x1 mm were used for the homogenates. Different application sites on the gel were tested to detect the most suitable position for each enzyme. For allozymes, the homogenates were initially prefocused at a constant power of 3 W for 30 min. The focusing was then continued at 5 W for 2 h and reached the final voltage of 850 V. For general proteins, the focusing started at a constant power condition of 5 W for 30 min. Power conditions were then increased to 8 W for 3 h to reach a final voltage value of 1450 V.

The following six enzymes were examined: esterase (EST, E.C. 3.1.1.2), hexokinase (HK, E.C. 2.7.1.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), malic enzyme (ME, E.C. 1.1.1.40), phosphoglucose isomerase (GPI, E.C. 5.3.1.9), and superoxide dismutase (SOD, E.C. 1.15.1.1). These enzymes were stained according to the procedures described by Brewer & Sing (1970), Shaw & Prasad (1970), Richmond & Powell (1970), Shows *et al.* (1970) and Vallejos (1983), and modified as suggested by Brockmeyer (1991) for annelids. General proteins were silver stained according to the method of Heukeshoven & Dernick (1983).

The following abbreviations are used in referring to various institutions : MNHN, Muséum National d'Histoire Naturelle, Paris; SMNH, Swedish Museum of Natural History, Stockholm; SZUO, Spezielle Zoologie, University of Osnabrück, Germany; ZMUC, Zoological Museum, University of Copenhagen, Denmark.

Results

Isozymes

The isozymes HK, MDH, ME (Fig. 2, 5) and SOD are monomorphic within each local population. EST and GPI are polymorphic. For EST (Fig. 3) there are at least two loci in the individual populations, one is monomorphic, the other is polymorphic but not interpretable. GPI has distinctly a more complex zymogram within local populations from France, U.K. and Sweden. The bands indicate that several alleles occur; however, these cannot unequivocally be assigned to loci. Therefore this enzyme is not useful for taxonomic discrimination of the individuals concerned here. No differences in band patterns were found between individuals from the same collection site which could be traced back to differences in sex and age.

The Swedish animals can easily be distinguished from those of France and the U.K. by exclusive band patterns at five isozymes already examined (Fig. 4-8). The presence or the absence of these "exclusive" bands are summarized in Table 2.

As there was a single specimen each from Norderney, Helgoland and Helsingør, which had a small body size, it was not possible to perform an analysis for the 6 enzymes and only the MDH was investigated. Their MDH band patterns were similar to that of the Swedish population (Fig. 9).

General protein patterns

The comparison of local populations and single specimens of "*Eulalia viridis*" with respect to general protein patterns after a non-specific silver staining under native conditions gave results similar to the isozyme studies. Patterns were reproducible (Fig. 10) within each population with a high degree of constancy, irrespective of sex (Fig. 12). General

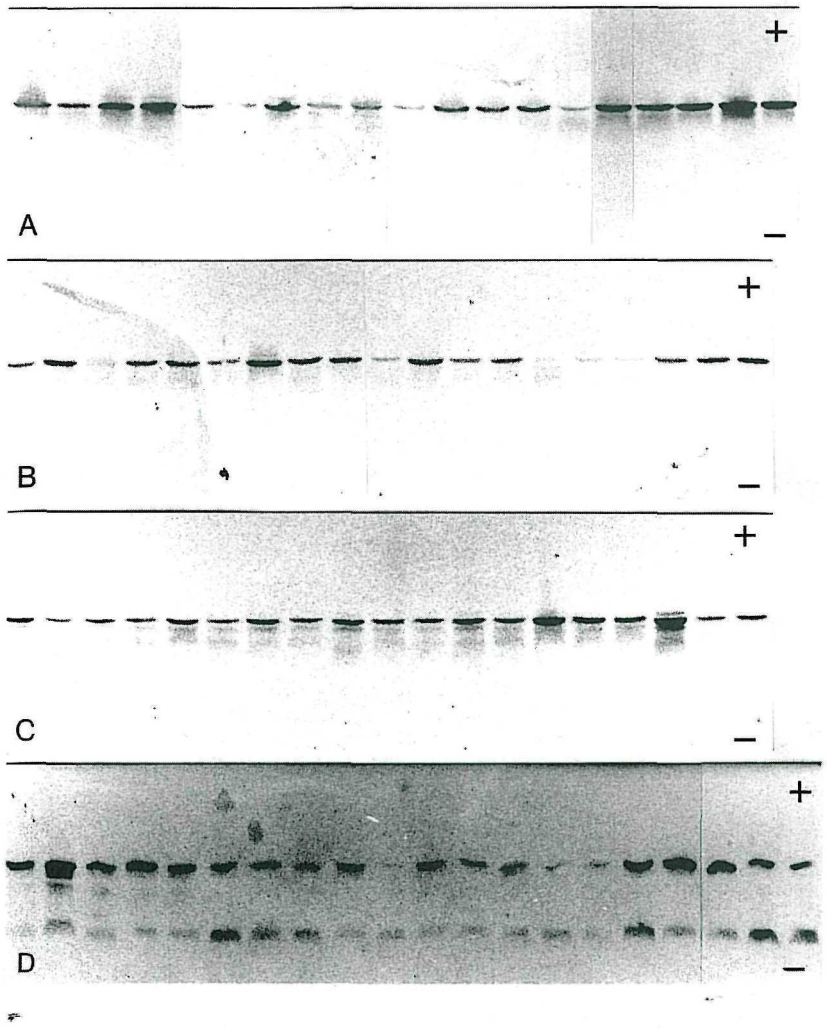

Figure 2: Isozyme patterns of *Eulalia* specimens from different sampling stations; proposed species name *Eulalia clavigera.* A: MDH of individuals from Saint-Efflam. B: MDH of individuals from Granville. C: MDH of individuals from Newcastle. D: ME of individuals from Granville.

Figure 2 : Profils électrophorétiques des isoenzymes de spécimens d'*Eulalia* provenant de différents sites d'échantillonnages ; nom d'espèce proposé *Eulalia clavigera.* A : MDH d'individus de Saint-Efflam. B : MDH d'individus de Granville. C : MDH d'individus de Newcastle. D : ME d'individus de Granville.

protein patterns represent a useful criterion to distinguish local populations of *"Eulalia viridis"* as polymorphism among individuals is restricted. The pattern of the Swedish animals differs distinctly from those of animals from France and the U.K. (Fig. 11). The general protein pattern of the Swedish population contains four "exclusive" bands while there are seven in the western local populations. The single specimens from Norderney, Helgoland and Helsingør show bands located in a similar position to those of the Swedish animals (Fig. 12). The smaller number of bands is due to the smaller size of these specimens.

The general protein pattern of another phyllodocid species, *Phyllodoce maculata*, has only a few bands that are identical to those in *Eulalia viridis* (Fig. 12).

Taxonomy

Eulalia viridis (Linnaeus, 1767)

Nereis viridis Linnaeus, 1767. Müller 1776. Fabricius 1780.

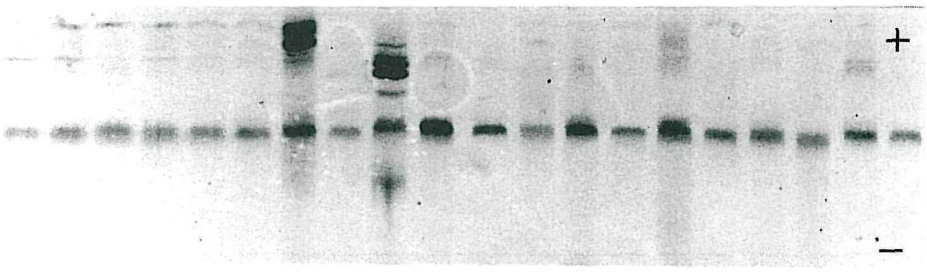

Figure 3: EST of individuals from Newcastle. Proposed species name: *Eulalia clavigera.*
Figure 3 : EST d'individus de Newcastle. Nom d'espèce proposé : *Eulalia clavigera.*

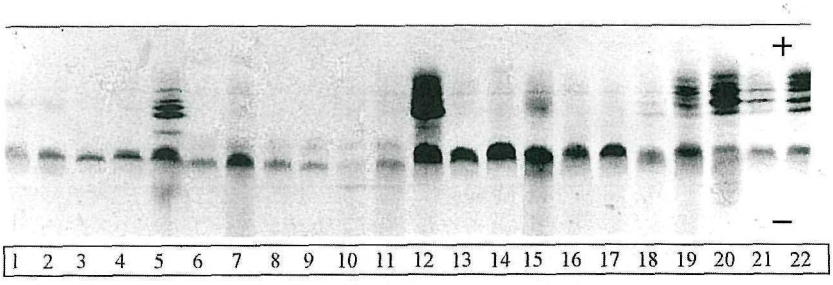

Figure 4: EST patterns of *Eulalia* specimens from different sampling stations. 1-5: Saint-Efflam; 6-11: Tjärnö; 12-17: Granville; 18-22: Newcastle.
Figure 4 : Profils électrophorétiques d'EST de spécimens d'*Eulalia* provenant de différents sites d'échantillonnages. 1-5 : Saint-Efflam ; 6-11 : Tjärnö ; 12-17 : Granville; 18-22 : Newcastle.

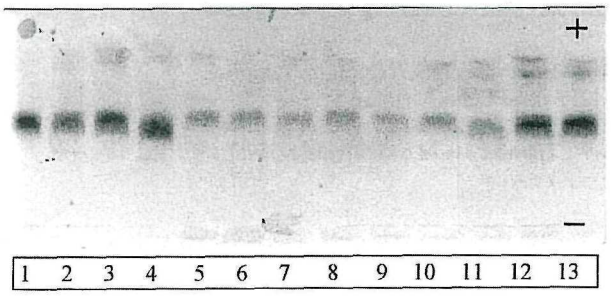

Figure 5: HK patterns of *Eulalia* specimens from different sampling stations. 1-4: Newcastle; 5-10: Tjärnö; 11-13: Granville.
Figure 5 : Profils électrophorétiques de HK de spécimens d'*Eulalia* provenant de différents sites d'échantillonnages. 1-4 : Newcastle ; 5-10 : Tjärnö ; 11-13 : Granville.

Eulalia viridis. Örsted 1843. Malmgren 1865. Bergström 1914. Hartmann-Schröder 1971 pars. Pleijel 1993 pars (Fig. 80).

Material examined: Neotype (ZMUC-POL-428) plus 64 other specimens (MNHN, 2 specimens; SMNH, 2 specimens; SZUO, 60 specimens) from Klinken (narrow sound between islands of Råssö and Saltö), south of Tjärnö, West Sweden, among tubes of *Pomatoceros triqueter*, 7 m, 24 Aug.-2 Sept. 1994, coll. S. Bonse; 14 specimens, 15 m, 31 July 1985, coll. D. Eibye-Jacobsen (ZMUC). About 300 specimens from Sweden, Denmark, Germany, Faroe Islands, Iceland and West Greenland (ZMUC).

Description: Neotype a complete specimen with 116 segments, length 36 mm, width including dorsal cirri 2.1 mm, excluding parapodia 1.3 mm. Size range of other specimens: 10-85 mm. Prostomium cordiform with a straight posterior margin, in neotype about 1 1/4 times as broad as long (Fig. 13 A), often more elongate. Five antennae, median antenna inserted halfway between eyes and the anterior margin of the prostomium. Dorsal pair of frontal antennae somewhat longer and thinner than the ventral pair, about 3/4 the length of the prostomium; median antenna slightly shorter and thinner. Two eyes, relatively large, diameter about one fifth of the prostomium width, reddish-brown with an anterolateral lens.

Proboscis not everted, but observed in several other specimens, long and clavate (Fig. 13 F), covered with diffusely distributed, conical papillae. Papillae gradually increase in size towards distal end, diameter ranging from 25-40 µm at the proximal end to 40-60 µm at the distal end (specimen length: 16-73 mm, n = 25). Opening of the everted proboscis surrounded by a ring of much larger, oral papillae, usually 14 in number (sometimes only 12 or as many as 28-30).

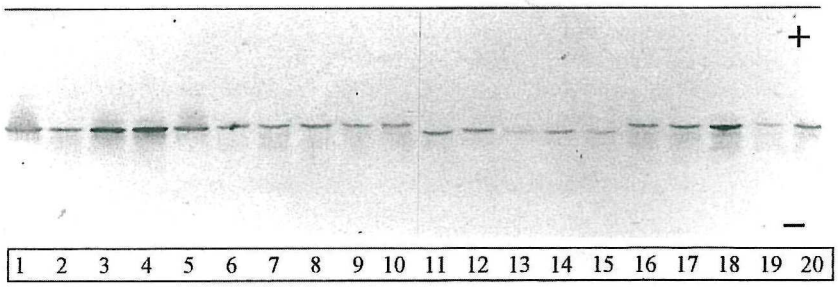

Figure 6: MDH patterns of *Eulalia* specimens from different sampling stations. 1-4: Saint-Efflam; 5-10: Granville; 11-15: Tjärnö; 16-20: Newcastle.

Figure 6 : Profils électrophorétiques de MDH de spécimens d'*Eulalia* provenant de différents sites d'échantillonnages. 1-4 : Saint-Efflam ; 5-10 : Granville ; 11-15 : Tjärnö ; 16-20 : Newcastle.

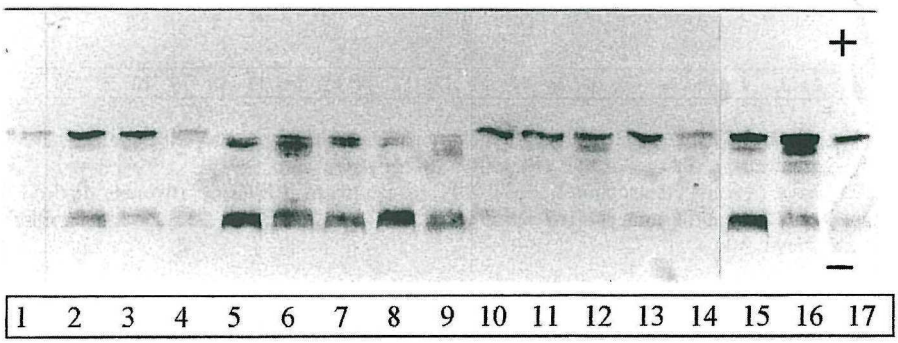

Figure 7: ME patterns of *Eulalia* specimens from different sampling stations. 1-4: Newcastle; 5-9: Tjärnö; 10-14: Granville; 15-17: Saint-Efflam.

Figure 7 : Profils électrophorétiques de ME de spécimens d'*Eulalia* provenant de différents sites d'échantillonnages. 1-4 : Newcastle ; 5-9 : Tjärnö ; 10-14 : Granville ; 15-17 : Saint-Efflam.

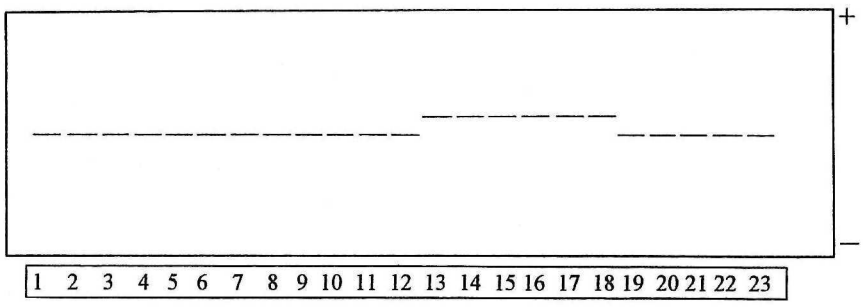

Figure 8: SOD patterns (traced) of *Eulalia* specimens from different sampling station. 1-6: Saint-Efflam; 7-12: Granville; 13-18: Tjärnö; 19-23: Newcastle.

Figure 8 : Profils électrophorétiques (tracés) de SOD de spécimens d'*Eulalia* provenant de différents sites d'échantillonnages. 1-6 : Saint-Efflam ; 7-12 : Granville ; 13-18 : Tjärnö ; 19-23 : Newcastle.

Table 2: Differentiation of the populations using the six enzymes investigated. Different numbers within the same horizontal row indicate different isozyme patterns. Identical numbers indicate that the populations cannot be differentiated by this enzyme.

Tableau 2 : Différenciation des populations à l'aide des six enzymes étudiés. Des chiffres différents dans une même rangée horizontale indiquent différents profils électrophorétiques. Des chiffres identiques indiquent que les populations ne peuvent pas être différenciées par l'enzyme correspondant.

Enzyme	Population Saint-Efflam	Population Granville	Population Newcastle	Population Tjärnö
EST	1	1	1	2
HK	1	1	1	2
MDH	1	1	1	2
ME 1	1	1	1	1
ME 2	1	1	1	2
SOD	1	1	1	2

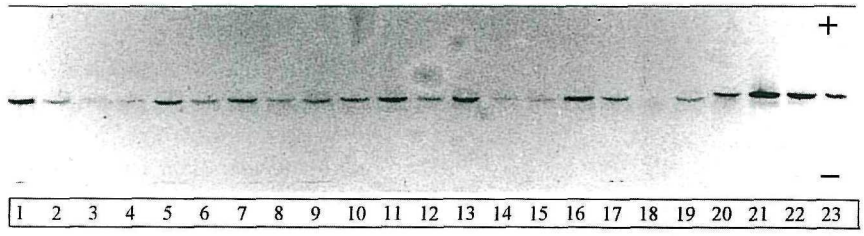

Figure 9: Assignement of questionable individuals from Norderney (17), Helsingør (18) and Helgoland (19) to the proposed species name *E. viridis* on the basis of agreement in MDH patterns of specimens from Tjärnö (1-16). 20-23: MDH patterns of specimens from Granville: proposed species name *E. clavigera*.

Figure 9 : Attribution au nom d'espèce proposé *E. viridis* d'individus incertains de Norderney (17), Helsingør (18) et Helgoland (19) sur la base d'une identité de profils électrophorétiques de MDH avec les spécimens de Tjärnö (1-16). 20-23 profils de MDH de spécimens de Granville : nom d'espèce proposé *E. clavigera*.

All the anterior segments distinct and fully developed. Tentacular cirri of segment 1 short, lanceolate, reach to segment 2-3. Ventral tentacular cirri of segment 2 of similar length, slightly to considerably broader and somewhat flattened. Dorsal tentacular cirri of segments 2 and 3 longer than the two other pairs, subulate, on neotype reaching segments 6 and 7, respectively, often slightly longer. Setae start on segment 3 ; a few setae may occur on segment 2 in juveniles.

The most anterior dorsal cirri are about twice as long as broad, broadly rounded at tip, becoming lanceolate by segment 8; by segment 20 the dorsal cirri are 2 1/2 -3 1/4 times as long as broad (Fig. 13 B); on segments of the middle part of the body the dorsal cirri are similar or more triangular, 2 1/4-3 times as long as broad (Fig. 13 C), slightly less on juveniles; on posterior segments more asymmetrical, 2 1/2-3 1/2 times as long as broad (Fig. 13 D). All dorsal cirri with well developed cirrophore, cirrus itself of almost uniform thickness from base to tip, with conspicuous pattern of "venation".

Ventral cirri oval in shape, about as long as neuropodium on middle segments, longer than neuropodium on anterior and posterior segments, more strongly tapered on posterior segments (Fig. 13 D).

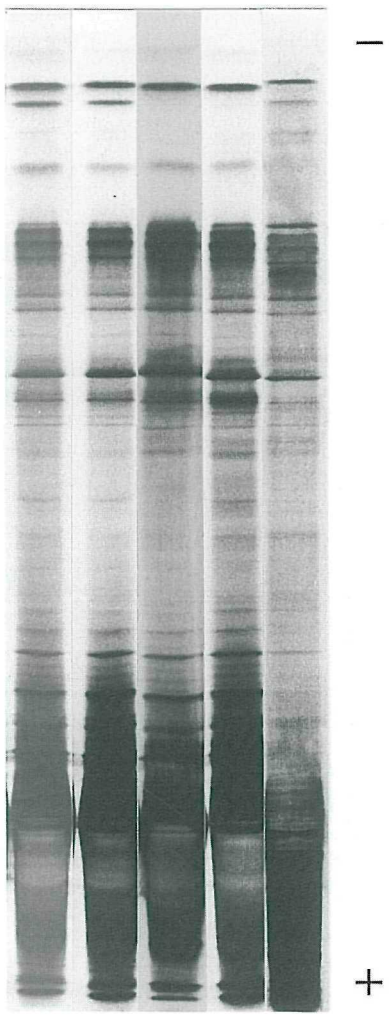

Figure 10: Reproducibility of the general protein patterns. Patterns of proteins of one specimen from Saint-Efflam (proposed species name *Eulalia clavigera*) on five different gels.

Figure 10 : Mise en évidence de la reproducibilité du profil électrophorétique général des protéines. Profils des protéines d'un spécimen de Saint-Efflam (nom d'espèce proposé *E. clavigera*) sur cinq gels différents.

Neuropodial presetal lamella well developed, divided into subequal supra- and subacicular lips by shallow cleft. Neuropodium with up to 35 compound spinigers. Rostrum of the setal shaft strongly inflated, covered by numerous teeth that increase in size towards end of shaft; two of the distal teeth (rarely only one) especially large, beak-like, separated by a gap of varying size. Setal blade very short and slender, with a somewhat drawn out tip.

Anal cirri missing on neotype, observed on many other specimens; subulate to slightly fusiform, 3-4 times as long as the basal width (Fig. 13 G).

Neotype uniformly orange-brown, with darker markings in front of eyes and dorsolaterally on segment 1. Dorsal and ventral cirri dark brown. The colour of other preserved specimens range from uniformly dark brown to light green or yellow. Live animals opaque yellowish green, sometimes with faint transverse, reddish-brown segmental bands; juveniles light green. Specimens in poor condition may turn reddish-brown before dying. Mature females dark yellowish green, mature males whitish green. Many specimens have a dark brown spot of pigment above and below each parapodium, which may disappear in alcohol.

Distribution: West coast of Sweden, Denmark, Germany, Norway (Malmgren 1865), Faroe Islands, Iceland, West Greenland to Godhavn (Disko Island). Shallow water species, reliable reports being from bottoms at depths from 5 to 150 m (uncommon at depths greater than 40 m). Found among serpulid tubes, in empty bivalve shells and on other hard substrates. *E. viridis* has often been reported from the eastern coast of Canada and the United States. Several preserved specimens from these areas were studied at the National Museum of Natural History, Washington D. C.; subtle differences appear to occur, but the animals were generally in poor condition. In light of the present investigation, careful study is needed to confirm whether the North American species is indeed *Eulalia viridis* (see also Eibye-Jacobsen 1991).

Eulalia clavigera (Audouin & Milne-Edwards, 1834)
Phyllodoce clavigera Audouin & Milne-Edwards, 1834.
Phyllodoce viridis (non Linnaeus, 1767). Johnston 1840.
Eulalia viridis (non Linnaeus, 1767). McIntosh 1908. Fauvel 1923. Hartmann-Schröder 1971 pars. Pleijel 1993 pars (fig. 79).

Material examined: Neotype (ZMUC-POL-429) plus 44 other specimens (MNHN, 2 specimens; SMNH, 2 specimens; SZUO, 40 specimens) from "Roche Rouge" at Saint-Efflam, Brittany, France, in clumps of *Mytilus edulis*, eulittoral, 21 June 1994, coll. S. Bonse; 31 spec. from Granville, Normandy, France, among green algae and aggregates of *Balanus* on rocks, eulittoral, 26 July 1994, coll. S. Bonse (SZUO); 27 spec. from near Newcastle-upon-Tyne, England, 4 km north of mouth of Tyne, in rock crevices, eulittoral, 22 July 1994, coll. S. Bonse (SZUO).

Description: Neotype a complete specimen with 134 segments, length 48 mm, width including dorsal cirri 4.0 mm, excluding parapodia 2.7 mm. Size range of specimens: 27-94 mm. Prostomium cordiform with a straight posterior margin, in neotype almost 1 1/2 times as broad as long (Fig. 14 A), often wider. Five antennae, median antenna inserted halfway between the eyes and the anterior margin of prostomium, sometimes closer to the eyes (e.g., on neotype). Dorsal pair of frontal antennae longer and thinner than the ventral pair, about 3/4 as long as prostomium ; median antenna similar or thinner and slightly shorter (neotype). Two eyes, diameter one fifth to one eighth

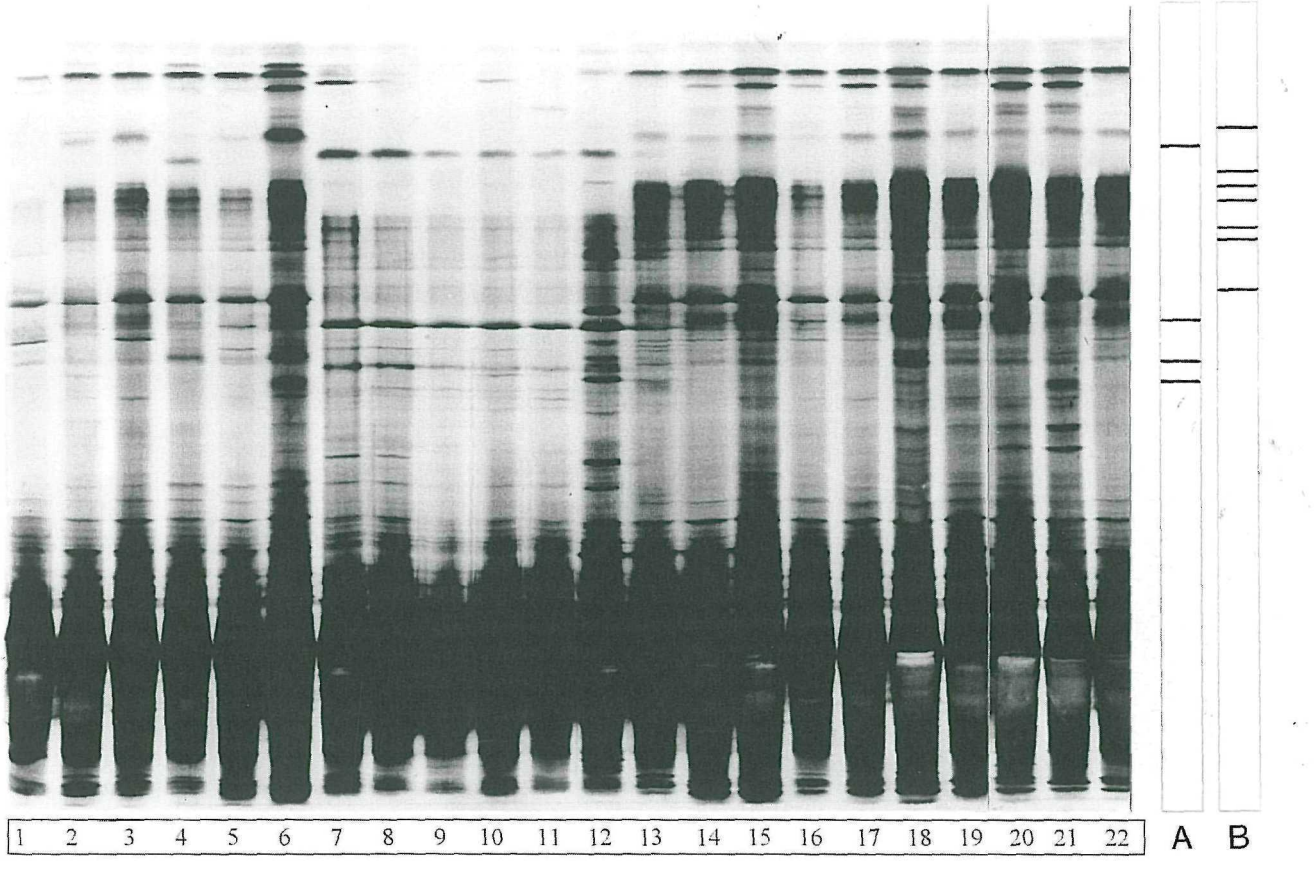

Figure 11: General protein patterns of *Eulalia* specimens from different sampling stations. Marked bands are "exclusive", appearing only in zymograms of the Tjärnö individuals (A) or only in those of the Newcastle, Saint-Efflam and Granville individuals (B). 1-6: Newcastle; 7-12: Tjärnö; 13-18: Granville; 19-22: Saint-Efflam.

Figure 11 : Profil électrophorétique de l'ensemble des protéines de spécimens d'*Eulalia* provenant de différents sites d'échantillonnages. Les bandes marquées sont "exclusives", apparaissant seulement dans des zymogrammes des individus de Tjärnö (A) ou seulement dans ceux de Newcastle, Saint-Efflam, Granville (D). 1-6 : Newcastle ; 7-12 : Tjärnö ; 13-18 : Granville ; 19-22 : Saint-Efflam.

of the prostomium width, reddish-brown, with an obvious anterolateral or central lens.

Proboscis everted but lost on neotype, observed in several other specimens, long and clavate, covered with diffusely distributed, conical papillae (Fig. 14 E). Papillae gradually increase in size towards the distal end, diameter ranging from 45-55 µm at the proximal end to 65-105 µm at the distal end (specimen length: 35-94 mm, n = 8). Opening of the everted proboscis surrounded by a ring of much larger, oral papillae, usually 14 in number (sometimes 28-30; 24 according to the original description).

All the anterior segments distinct and fully developed. Tentacular cirri of segment 1 short, slightly fusiform, reach to segment 2-3. Ventral tentacular cirri of segment 2 of similar length, broader and slightly flattened. Dorsal tentacular cirri of segments 2 and 3 longer than the two other pairs, subulate, on neotype reaching segments 7 and 8, respectively, often slightly shorter. Setae begin from segment 3 on all the specimens observed.

The most anterior dorsal cirri about 1 1/2 times as long as broad, broadly rounded, becoming elongate triangular by segment 10; on segment 20 strongly asymmetrical, twice as long as broad (Fig. 14 B; note, length measured from ventral point of cirrophore attachment to tip); on the middle segments less asymmetrical, 1 3/4-2 1/4 times as long as broad (Fig. 14 C); on posterior segments almost symmetrical, more elongate, about 2 1/2 times as long as broad (Fig. 14 D). All the dorsal cirri with a well developed cirrophore, cirri on the middle part of the body very thick at base, gradually tapering towards the tip, pattern of "venation" not always obvious.

Ventral cirri oval, usually clearly shorter than neuropodium, less broad on posterior segments (Fig. 14 D).

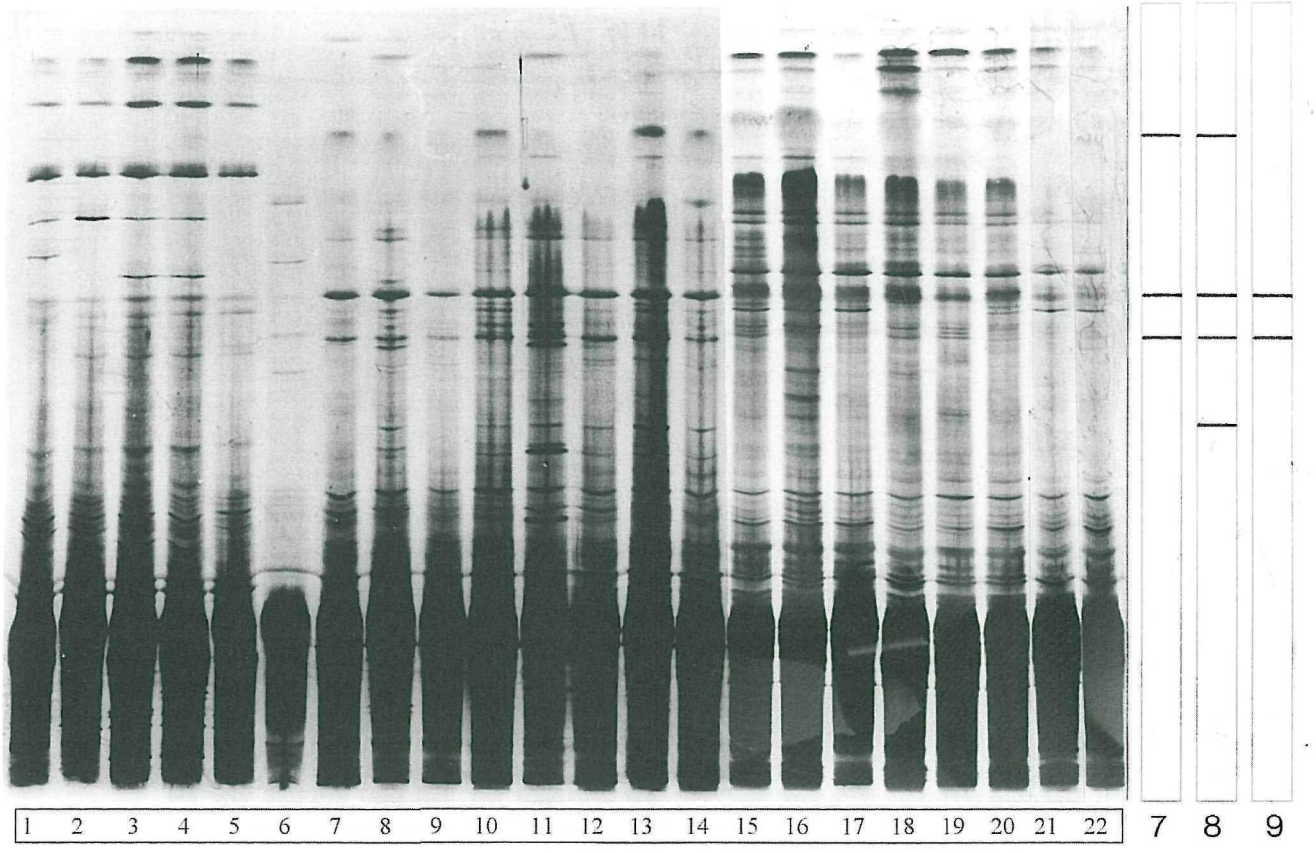

Figure 12: Proposed assignement of questionable individuals to the species *E. viridis* on the basis of agreement in general protein patterns. For comparison, others species not closely related to *E. viridis* are shown. 1-5: *Phyllodoce maculata* (Tjärnö); 6: *Eulalia bilineata* (Tjärnö); 7: *Eulalia viridis* (Norderney); 8: *Eulalia viridis* (Helgoland); 9: *Eulalia viridis* (Helsingør); 10-14: *Eulalia viridis* (Tjärnö); 15-22 specimens of the proposed species name *Eulalia clavigera*; 15-17: males from Granville; 18-20: females from Granville, 21-22: females from Newcastle. Bands marked are present in zymograms of all *E. viridis*-individuals from Tjärnö and in those of single individuals from Norderney (7), Helgoland (8) and Helsingør (9), but not in the zymograms of individuals of *E. clavigera* from Granville and Newcastle.

Figure 12 : Attribution proposée d'individus incertains à l'espèce *E. viridis* sur la base de similitudes dans le profil électrophorétique de l'ensemble des protéines. Pour comparaison, d'autres espèces non étroitement apparentées à *E. viridis* sont montrées. 1-5 : *Phyllodoce maculata* (Tjärnö) ; 6 : *Eulalia bilineata* (Tjärnö) ; 7 : *Eulalia viridis* (Norderney) ; 8 : *Eulalia viridis* (Helgoland) ; 9 : *Eulalia viridis* (Helsingør) ; 10-14 : *Eulalia viridis* (Tjärnö) ; 15-22 spécimens attribués à l'espèce proposée *Eulalia clavigera* ; 15-17 : mâles de Granville ; 18-20 : femelles de Granville, 21-22 : femelles de Newcastle. Les bandes dessinées sont présentes dans les zymogrammes de tous les individus de *E. viridis* de Tjärnö et dans les individus isolés de Norderney (7), Helgoland (8) et Helsingør (9), mais pas dans les zymogrammes des individus attribués à *E. clavigera* de Granville et Newcastle.

Neuropodial presetal lamella well developed, clearly divided into supra- and subacicular lips by a rather deep, narrow cleft (when viewed at the optimal angle). Neuropodium with up to 50 compound spinigers. Morphology of the setae as in *E. viridis*.

Anal cirri missing on neotype, observed on a few other specimens; subulate, 2-3 times as long as wide at base (Fig. 14 F).

Neotype creamy green on dorsum and venter, with brown cirri and parapodia. Dorsum may also be greenish-brown, with a metallic sheen (as mentioned in original description), or uniformly brown. Prostomium, tentacular cirri and dorsal cirri often with dark brown markings. Many specimens with prominent dark spots lateral to the eyes (these lateral spots were called eyes in the original description; corrected in Johnston 1840, although under the name of *Phyllodoce viridis*). Live animals deep emerald green in colour, mature males lighter, juveniles light green. A few animals with dark spots below the parapodia.

Distribution: *Eulalia clavigera* has only been reported from France and Great Britain. Its range probably includes the Atlantic coast of the Iberian Peninsula and Madeira.

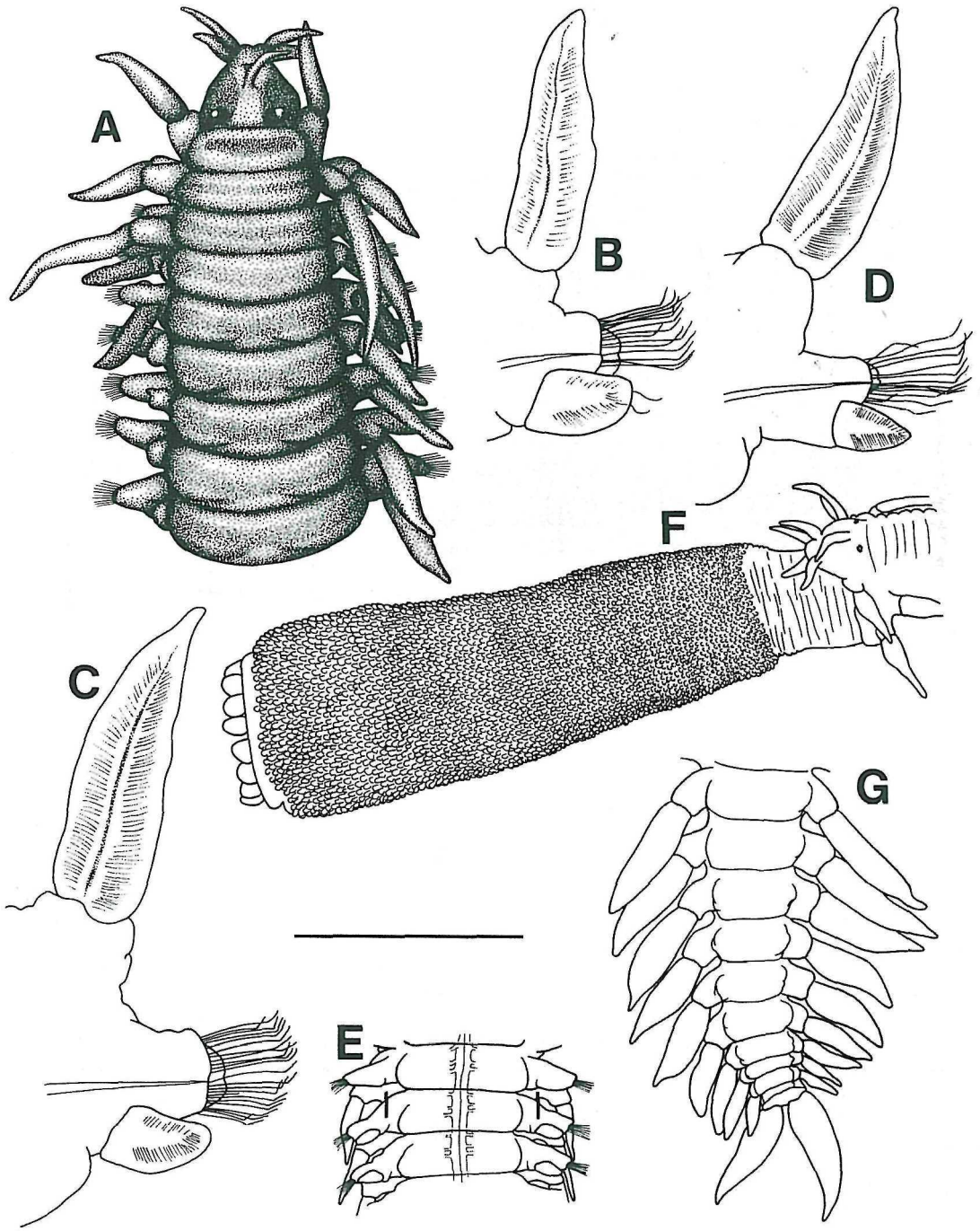

Figure 13: *Eulalia viridis* (Linnaeus, 1767). A-E Neotype; F specimen from Godhavn, Disko Island, West Greenland; G specimen from Reykjavik, Iceland. A: anterior end, dorsal view. B: parapodium of segment 20, posterior view. C: parapodium of segment 59, posterior view. D: parapodium of segment 96, posterior view. E: segments 49-51, ventral view; distance between thick lines indicates venter width, as used in this paper and refers to segment 49, the uppermost of the segments shown. F: anterior end with everted proboscis, dorsolateral view. G: posterior end with anal cirri, dorsal view. Scale = 1 mm (A, G), 0.5 mm (B-D), or 2 mm (E, F).

Figure 13 : *Eulalia viridis* (Linnaeus, 1767). A-E Neotype ; F spécimen de Godhavn, Ile de Disko, Ouest Groenland ; G spécimen de Reykjavik, Islande. A : extrémité antérieure vue dorsale. B : parapode du segment 20, vue postérieure. C : parapode du segment 59, vue postérieure. D : parapode du segment 96, vue postérieure. E : segments 49-51, vue ventrale ; la distance entre les 2 traits indique la largeur du corps telle qu'elle est mentionnée dans cet article et se rapporte au segment 49, le plus antérieur des trois segments montrés. F : extrémité antérieure avec la trompe sortie, vue dorso-latérale. G : extrémité postérieure avec les cirres pygidiaux, vue dorsale. Echelle = 1 mm (A, G), 0.5 mm (B-D), ou 2 mm (E, F).

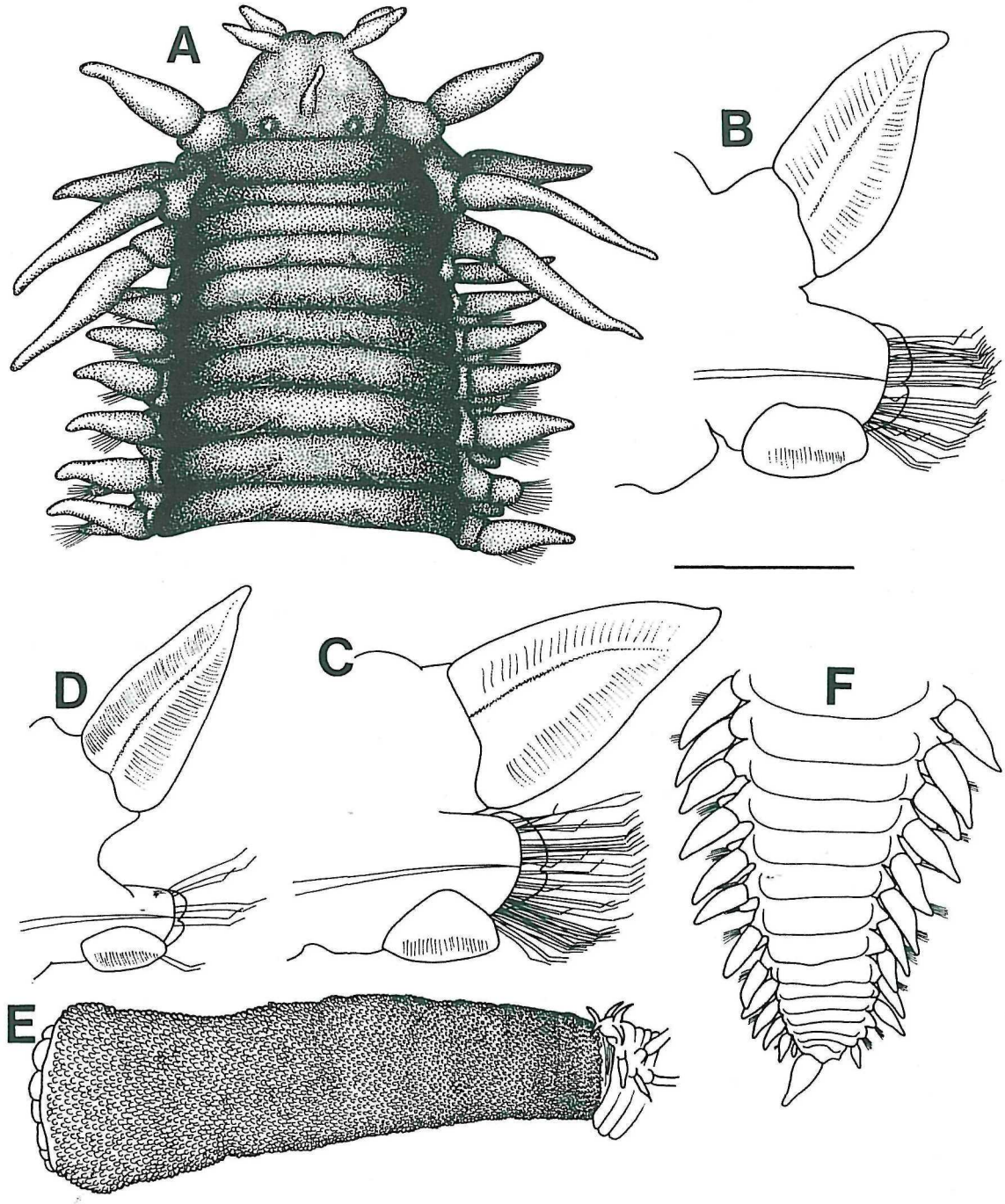

Figure 14: Specimens belonging to the proposed species name *Eulalia clavigera* (Audouin & Milne-Edwards, 1834). A-D Neotype from Saint-Efflam; E, F other specimens from same sample. A: Anterior end, dorsal view. B: parapodium of segment 22, posterior view. C: parapodium of segment 59, posterior view. D: parapodium of segment 108, posterior view. E: anterior end with everted proboscis, ventrolateral view. F: posterior end with anal cirri, dorsal view (right anal cirrus missing). Scale = 1 mm (A, F), 0.5 mm (B-D), or 4 mm (E).

Figure 14 : Spécimens attribués à l'espèce proposée *Eulalia clavigera* (Audouin & Milne-Edwards, 1834). A-D Neotype de Saint-Efflam ; E, F autres spécimens du même échantillon. A : extrémité antérieure, vue dorsale. B : parapode du segment 22 vue postérieure. C : parapode du segment 59, vue postérieure. D : parapode du segment 108, vue postérieure. E : extrémité antérieure avec la trompe sortie, vue ventro-latérale. F: extrémité postérieure avec un cirre pygidial, vue dorsale, (le cirre droit manquant). Echelle = 1 mm (A, F), 0.5 mm (B-D) ou 4 mm (E).

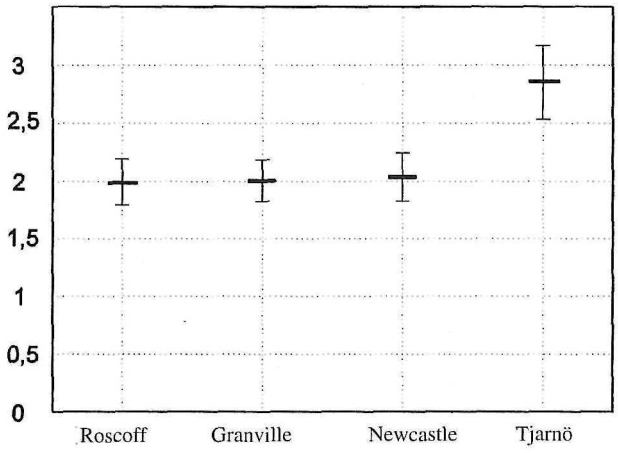

Figure 15: Comparison of the dorsal cirri of individuals from four different sampling stations. Mean value of the length to width ratio (broad bars) and standard deviations (thin bars). It is proposed that specimens from Saint-Efflam, Granville, and Newcastle belong to the species *Eulalia clavigera*, those from Tjärnö to the species *E. viridis*.

Figure 15 : Comparaison des cirres dorsaux d'individus provenant de quatre sites différents. Valeurs moyennes du rapport longueur sur largeur (traits larges) et écarts-types (traits fins). Il est proposé d'attribuer les spécimens de Saint-Efflam, Granville, et Newcastle à l'espèce *Eulalia clavigera*, et ceux de Tjärnö à l'espèce *E. viridis*.

Various reports of *E. viridis* from the Mediterranean Sea may refer to this species. However, specimens labelled as *E. viridis* in ZMUC belong to another, possibly undescribed species of *Eulalia* (pers. obs.). *E. clavigera* is only known from shallow depths, living in habitats similar to those of *E. viridis*, e.g., hard substrates covered with bivalves, barnacles or serpulid tubes, as well as in rock crevices.

Remarks on morphological characters

Linnaeus (1767) probably did not designate type specimens for *Eulalia viridis* (neither found at SMNH nor at the Natural History Museum, London). The type locality is referred to as "in Oceano septentrionale" (in the northern ocean). Similarly, Audouin and Milne-Edwards (1834) do not appear to have designated type material for *E. clavigera* (neither found at the MNHN nor at the Université Catholique, Angers, France). The type locality is "Vendée et Manche", i.e., the west coast of France between St. Nazaire and La Rochelle and the northern coasts of Brittany and Normandy.

As it became clear during this study that two species were probably involved, the question arose as to which one should bear the name *viridis*. Animals from the Faroe Islands, Iceland and West Greenland clearly belong to the species represented by the Swedish population. It is more likely that "the northern ocean" referred to these areas, rather than the coasts of France and England. Therefore, *Eulalia viridis* is used here for the species found in Sweden. Another reason why this choice was made is that a valid name, *Eulalia clavigera*, is available for the species from France and England, which is not the case for the species from Sweden.

Apart from differences due to ontogenetic changes, there is a considerable amount of variation among individuals of *Eulalia viridis*, especially with regard to the degree of elongation of the dorsal cirri. Average values of the length/width ratio of dorsal cirri from median segments are significantly different from those of *Eulalia clavigera* (Fig. 15) where the dorsal cirri are usually also distinctly more asymmetrical. However, some specimens are not easily identified using these characters.

Likewise, the ventral cirri of *E. viridis* are clearly longer relative to the neuropodia than in *E. clavigera*. However, more specimens have to be studied in order to confirm this difference, especially juveniles of *E. clavigera* (which were not present in our material).

Other diagnostic differences relate to the length/width ratio of the prostomium, the size of the eyes relative to the width of the prostomium, the length of the ventral cirri relative to that of the neuropodia and the length/width ratio of the anal cirri. All these relationships show a varying degree of overlapping and could not be diagnostic on their own.

The ratio between venter width, defined as the distance between the bases of the two neuropodia of segment 50 (Fig. 13 E), and the greatest length of the dorsal cirri from that segment (removed from the body and lying completely flat) provides a more reliable biometric way to distinguish *Eulalia viridis* from *E. clavigera*. In the former, this ratio usually lies between 1.2 and 1.5, a few specimens showing values as high as 1.77 (n = 17, means = 1.45, standard deviation = 0.18). In *E. clavigera*, this value ranged from 1.8 to 2.0 in most animals (2.5 in the largest specimen), with two values falling below this range (n = 9, means = 1.94, standard deviation = 0.33). The Mann-Whitney U-test supports the hypothesis that the two means are significantly different (U = 11, which is significant at the 1 % level). The venter width/dorsal cirral length ratio is much more reliable than the equivalent ratio using the width of the dorsum. The latter is more dependant on the coincidental swelling which often takes place when animals are preserved, whereas the width of the venter seems to be more constant, correlated with the presence of the strong ventrolateral, longitudinal muscle bands.

A far more dependable biometric method of distinguishing *Eulalia viridis* from *E. clavigera* is by measuring the ratio between the length and the thickness of midbody dorsal cirri (note, thickness as opposed to width, i.e., perpendicular to the plane of flattening of the cirrus).

Length is measured as the extended length of the dorsal cirrus removed from the body, and thickness is measured as its maximum value (i.e., just above the base of the cirrus). The ratio varies from 4.9 to 5.9 in *E. viridis* (n = 25, means = 5.36, standard deviation = 0.26) and from 3.0 to 3.5 in *E. clavigera* (n = 9, means = 3.29, standard deviation = 0.15). Thus, this index appears to separate the two species quite well and it is also valid for juveniles (at least in *E. viridis*).

Finally, the most reliable diagnostic character is the size of the proboscideal papillae. In *E. viridis*, papillae located on the proximal part of the proboscis display a maximum diameter of 40 µm. In *E. clavigera*, the minimum diameter of these papillae is 45 µm. Similarly, the papillae on the distal part of the proboscis have a maximum diameter of 60 µm in *E. viridis*, whereas the minimum diameter in *E. clavigera* is 65 µm (and in most cases considerably larger).

Discussion

Enzyme electrophoresis is known to be a powerful method to separate polychaete sibling species belonging, for instance, to the genera *Capitella, Glycera, Polydora, Nereis, Nephtys* and *Marenzelleria* (Grassle & Grassle 1976, Nicklas & Hoffmann 1979, Mustaquim 1988, Fong & Garthwaite 1994, Schmidt & Westheide 1994, Manchenko & Radashevsky 1994, Bastrop et al. 1995) and is therefore appropriate for examing *Eulalia*.

Isoelectric focusing which was used in the present analysis, offers certain advantages over other electrophoretic techniques. The discriminative patterns are highly reproducible and, in contrast, e.g., to the conventional PAGE method, can be compared directly with one another (see also Schmidt & Westheide 1994). Because a standard sample is used, zymograms of single individuals could be compared on different gels, allowing slight differences in the positions of bands to be detected.

An advantage of the general protein IEF, in comparison with the enzyme IEF, is that even single individuals can be evaluated in terms of their very complex protein patterns. Thus the individual animals from Norderney, Helgoland and Helsingør could be unequivocally assigned to the Swedish type using their general protein pattern (Fig. 12). Furthermore, with general protein IEF the likelihood of finding diagnostic differences between different taxa is greater than with the IEF of a few enzymes, because the general protein patterns show a large number of different water-soluble proteins, including non-enzymatic ones. The general protein band patterns reveal 11 markers that do discriminate between the *Eulalia* individuals recognized here as two separate species (Fig. 11).

The silver staining used here (Heukeshoven & Dernick 1983) is extremely sensitive and can stain proteins even at low concentrations. The separation of numerous water-soluble proteins is widely employed in studies on food chemistry and in plant conservation (Stegemann *et al.* 1982). In agriculture, general-protein IEF also makes an important contribution, as it enables a precise diagnosis of strains of grasses and cereal crops (Hahn *et al.* 1994) and is also used to test the outcome of breeding trials (Hahn *et al.* 1994). Although general-protein studies are not yet commonly used in zoological taxonomy, they have been found to be very helpful in this field (Bylund & Djupsund 1977, Stegemann *et al.* 1982, Guérin & Kerambrun 1984, Carmona *et al.* 1989, Brockmeyer 1991, Westheide & Brockmeyer 1992, Schmidt & Westheide 1994).

On the basis of both the isoenzyme and the general protein patterns, the local populations of *Eulalia* studied here can be subdivided into a western group (Saint Efflam, Granville, Newcastle) and an eastern group (Norderney, Helgoland, Helsingør, Tjärnö).

Apart from one gene locus, ME-1, no alleles are shared between individuals from France/England and the north-eastern sampling stations. The results of isozyme and general protein studies show a large number of genetic markers which discriminate between the eastern and the western group (Table 2). It has been shown that in some cases, species (e.g. on the genus *Enchytraeus*), separated by cross-breeding experiments differ only in the position of a few bands either among a large number of tested isozymes or their general protein patterns (Brockmeyer 1991, Westheide & Brockmeyer 1992); hence it is surprising that the divergence observed between *Eulalia viridis* and *E. clavigera* is so great.

A statistical evaluation of the present protein data by means of genetic identity values and genetic distances was found to be unsuitable. Four of the six isozymes examined - and accordingly their alleles - were fixed within each species and differences are therefore diagnostic for *E. clavigera* and *E. viridis*. As a consequence there is no need to calculate genetic identities or genetic distances.

The identities between the English and the French individuals may be ascribable to the drifting of larvae across the Channel and along the coasts in ocean currents, since the free-swimming trochophore stage stays two to three weeks in the water column prior to the first bottom-dwelling stage (Husemann 1992). However, why do the western (i.e. *E. clavigera*) and eastern (i.e. *E. viridis*) populations differ in this study? One explanation is that larvae of populations of the original common species separated after the last Ice Age are no longer able to cross the North Sea barrier (or, apparently, the Faroe-Shetland Channel) (see Otto (1983) for the prevailing currents in the North Sea). It is also conceivable that while the Ice Age was still in progress, populations became reproductively isolated, giving rise to distinct species.

Unequivocal differences between the western (England, France) and eastern (Sweden) populations have been detected by the method of isoelectric focusing, and closer scrutiny in morphology has also revealed differences. The dorsal cirri in the anterior and middle parts of the body of specimens from England and France have a more pronounced ventral bulge, thus being more asymmetrical, than those of specimens from Sweden. These latter are also distinguished by a lighter colouration in adult individuals. However, the slight variation in the shape of the dorsal cirri (6% of the observed individuals could not be identified with certainty) makes difficult any individual assignment to a species by using this character alone. The problem with colouration is that it changes during development and is difficult to use (as a criterion) in young individuals species identification. The dorsal cirri are significantly thicker in *E. clavigera* than in *E. viridis*, at least in adults, but this character has not yet been studied on juveniles in the former species. The proboscideal papillae also differ in size for both species, but the use of this character demands precise measurements and should be considered together with the size of the animal (see above). Furthermore, if the proboscis is not everted, dissection must be performed. Hence, molecular markers remain important in future taxonomic studies of the *Eulalia viridis* complex whenever the origin of a specimen is uncertain, the animal is not fully developed or it is collected in additional localities.

The fact that the two groups exhibit different reproductive breeding periods also supports the conclusion that two species are involved. Both are polytelic and reproduce once a year (Husemann 1992). Sexually mature individuals of *E. clavigera* can usually be found in July and August on the coast at Saint-Efflam (Frank Gentil, pers. comm.), which also corresponds to the reproduction period occurring in Newcastle (Olive 1975). In Helgoland, Denmark and Sweden reproduction takes place somewhat earlier. In Helgoland pelagic larvae of *E. viridis* have been trapped generally from the beginning of May to the middle of June, and sexually mature animals from February to May (Gillandt 1979). Animals in the Øresund reproduce in June and July (pers. observation of D. Eibye-Jacobsen) and those in Sweden during May and June (Pleijel 1993). Several specimens from Iceland, collected in April, contained almost mature eggs. The reproductive period of *E. viridis* thus appears to begin 1 to 2 months before that of *E. clavigera*. If we consider an approximate duration of 4-6 weeks of the breeding period (Olive 1975, Husemann 1992) with some variation from year to year, it may overlap with the reproductive period of *E. clavigera* by a few days at the most. The different reproductive seasons thus corroborate the existence of a restricted gene flow or its complete absence between, e.g., the English and Swedish populations ; which may have lead to speciation in the genus *Eulalia*. Strangely, however, this pattern of different reproductive periods of the two species does not fit for the *Eulalia viridis* from the Plymouth area for which animals with eggs are recorded in May and June (Marine Biological Association 1957).

Acknowledgements

We are very grateful to Dr. Frank Gentil (Biological Station Roscoff, France), Dr. Peter Garwood (Biological Laboratory Newcastle upon Tyne, U.K.) and Dr. Fredrik Pleijel (Marine Biological Laboratory Tjärnö, Sweden), who helped us to collect the animals. We also thank Brigitte Rubæk (ZMUC) for the drawings of figures 13 and 14.

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