Identification of selected siderophores in the Baltic Sea environment by the use of capillary electrophoresis*

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Abstract

Extracts from seawater and sediment pore water samples were characterised by capillary electrophoresis (CE). Siderophores of the ferrioxamine family were identified. Ferrioxamine E is the dominant siderophore in both seawater and sediment pore water samples from different regions of the Baltic Sea. Ferrioxamine G was

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identified in subsurface seawater samples from the Gdańsk Deep. Rhodotorulic acid was also identified in seawater samples from the euphotic zone (0–30 m) of Puck Bay and in sediment pore water from Puck Bay and the Bornholm Deep. Ferrioxamine B was not found. The presence of catechol siderophores was not investigated.

1. Introduction

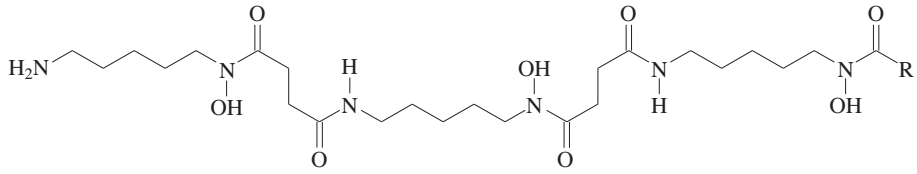
The concentration of iron ions in seawater is very low, ranging from 0.02 to 1.0 nmol dm^{-3} (Martin & Fitzwater 1988, Bruland *et al.* 1994) and is much lower than that required by most aquatic marine organisms (Sunda & Huntsman 1995).

As established recently, iron limitation can control rates of phytoplankton productivity and biomass in high-nutrient/low-chlorophyll (HNLC) regions of the world's oceans (Martin *et al.* 1994, Coale *et al.* 1996, Butler 1998). In seawater iron exists predominantly in the form of insoluble ferric hydroxide complexes $Fe(OH)_n$ which are not readily available for assimilation. Numerous marine micro-organisms have evolved a mechanism to acquire iron: they produce siderophores to solubilise and sequester iron(III). Siderophores are low-molecular-weight ligands that are synthesised by the majority of micro-organisms under conditions of low-iron stress (Neilands 1995). Siderophores have very high Fe(III) affinity constants (10^{25} to 10^{52}) and their synthesis is regulated by the level of iron concentration in the environment (Matzanke *et al.* 1989, Crumbliss 1991).

The majority of siderophores can be classified into two groups – hydroxamate or phenolate/catecholate (derivatives of 2,3-dihydroxybenzoic acid). Other chelating moieties found in siderophore structures include residues of α-hydroxy acids, carboxylic acids and oxazolines (Matzanke 1991). Hydroxamate siderophores are present in *Streptomyces* and *Actinomycetes* species, fungi and some enterobacteria. Bacterial siderophores from the

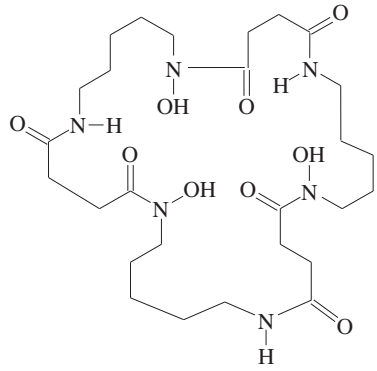
Table 1. Bacterial siderophores from the ferrioxamine family

Siderophore	Producing organism
Ferrioxamine (A_1, A_2, B, C, D_1, D_2, E, F, G, H)	*Streptomyces pilosus*, *S. griseus*, *S. griseoflavus*, *S. olivaceus*, *S. aureofaciens*, *S. galilaeus*, *S. lavendule*
Ferrioxamine B	*Arthrobacter simplex*
Ferrioxamine E, D_2, B	*Erwinia herbicola*
Ferrioxamine E	*Pseudomonas stutzeri* *Chromobacterium violaceum*
Ferrioxamine G	*Hafnia alvei*

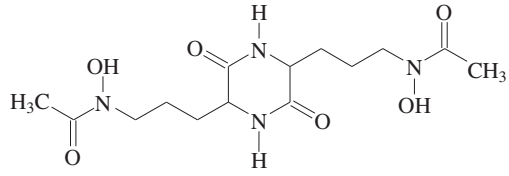


Desferrioxamine B R = CH_3

Desferrioxamine G R = $(CH_2)_2COOH$



Desferrioxamine E, Nocardiamine



Rhodotorulic acid

Fig. 1. Structure of hydroxamate siderophores

family of ferrioxamines and the producing organisms are listed in Table 1 (Matzanke 1991). These ferrioxamines occur as both linear and cyclic compounds. Whereas ferrioxamine E (nocardamine) and D_2 are cyclic compounds, all other ferrioxamines are linear. Rhodotorulic acid is a dioxypiperazine of N-δ-hydroxy-N-δ-acetyl-L-ornithine, which mediates iron uptake in the *Rhodotorula* yeast species (Matzanke 1991). The structures of these siderophores are shown in Fig. 1. The results of recent studies suggest that up to 99% of iron in the open ocean and coastal waters may be complexed by organic ligands. The stability constants of these ligands are comparable to synthetic desferal (Rue & Bruland 1995, van den Berg 1995, Wu & Luther 1995). Siderophore-like substances appear in the marine environment but little is known about their chemical composition. Only a few structures

of siderophores isolated from marine bacteria have been fully identified. Takahashi *et al.* (1987) isolated and characterised bisucaberin, which is produced by the deep-sea mud bacterium *Alteromonas haloplanktis*. This molecule is a cyclic dimer of N-succinyl-N-hydroxycadaverine and is closely related to ferrioxamine E, the trimer of the same moiety. Alterobactin A and B from the heterotrophic marine bacteria *Alteromonas luteoviolacea* were identified by Reid & Butler (1991) and Reid *et al.* (1993), aerobactin from oceanic *Vibrio* species was identified by Haygood *et al.* (1993) and anguibactin produced by the fish pathogen *Vibrio anguillarium* was characterised by Jalal *et al.* (1989).

Strains of open-ocean bacteria from the genera *Vibrio*, *Alteromonas*, *Alcaligenes*, *Pseudomonas* and *Photobacterium* and fish-pathogenic bacteria from the Baltic Sea produce hydroxamate and catecholate-type siderophores (Trick 1989, Gierer *et al.* 1992, Wilhelm & Trick 1994, Lewis *et al.* 1995). Marine and freshwater phytoplankton grown at low iron levels produce hydroxamate-, catecholate- and atypical-type siderophores (Estep *et al.* 1975, Trick *et al.* 1983, Kerry *et al.* 1988, Wilhelm 1995, Wilhelm & Trick 1995, Wilhelm *et al.* 1996) but only one was structurally identified as schizokinen, produced by the cyanobacterium *Anabaena* sp. (Simpson & Neilands 1976).

Siderophores complex iron and promote iron transport to the cells which excreted them. Murphy *et al.* (1976) have shown that during cyanobacterial blooms, the growth of other algae can be completely suppressed owing to iron deficiency, since they are unable to use the usual mechanism of iron transport into cells. Siderophores display a specific activity not only in relation to the organisms from which they originate. Siderophores such as rhodotorulic acid, retro-(Et)-arthrobactin, schizokinen, desferrioxamine B and DHB (2,3-dihydroxybenzoic acid) can modify physiological processes in populations of cyanobacteria and green algae cells (Kosakowska & Falkowski 1994, Surosz & Kosakowska 1996).

In this work we have characterised four siderophores in seawater samples from different regions of the southern Baltic Sea. Capillary electrophoresis was used to separate siderophores in seawater by a recent method (Mucha *et al.* 1999).

2. Methods

2.1. Sample collection and treatment

Water and sediment samples were collected during cruises of the r/v 'Oceania' in the southern Baltic Sea (Table 2, Fig. 2). Samples of surface and subsurface seawater were collected with a bathometer, samples of

Table 2. Position of Baltic Sea stations

Station	Name	Coordinates	Seawater type	Volume [dm^3]	Sampling date
GD	Gdańsk Deep	54°34′N	surface	32.0	05.96
		19°10′E	subsurface	45.0	05.96
			near bottom	2.8	04.97
BD	Bornholm Deep	54°50′N	surface	44.0	05.96
		15°22′E	subsurface	41.0	05.96
		55°09′N	sediment pore water	0.5	04.97
		15°55′E			
SFW	Słupsk Furrow	55°15′N	surface	50.0	05.96
	West	17°13′E	subsurface	45.0	05.96
PB	Puck Bay	54°34′N	sediment pore water	0.8	08.96
		18°40′E	from 0 to 30 m	83.0	02.98
SFE	Słupsk Furrow	55°17.5′N	from 0 to 30 m	40.0	05.98
	East	18°00′E			

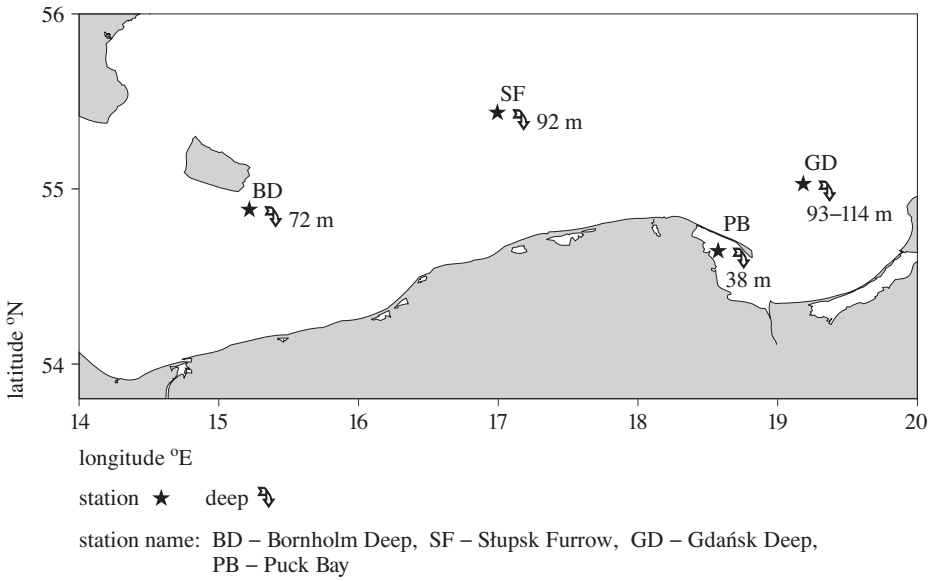


Fig. 2. The Baltic Sea – sampling stations

near-bottom seawater and sediment with a Nemistö corer. Pore water was obtained by centrifuging the sediment in 50 cm^3 polyethylene tubes at 4200 g for 30 min. The resulting supernatant was carefully pipetted off and used

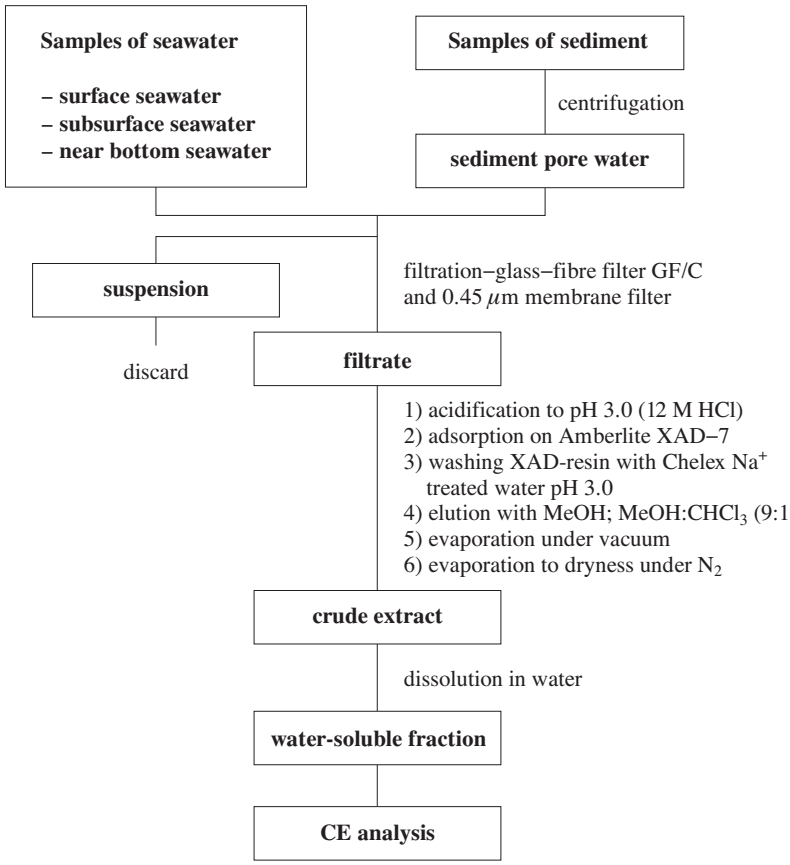


Fig. 3. Diagram of the procedure for the isolation of siderophores

after filtration (Carr & Chapman 1995). A diagram of the procedure for siderophore isolation adopted from the literature (Trick *et al.* 1983, Wilhelm & Trick 1994) is shown in Fig. 3.

2.2. Siderophore samples

Desferrioxamine B was purchased from Ciba-Geigy, Basle, Switzerland, in the form of desferrioxamine methansulphonic (Desferal). Ferrioxamine E and G were a gift from Dr. R. Reissbrodt (Robert Koch Institute, Wernigerode, Germany). Rhodotorulic acid was purchased from Sigma-Aldrich Fine Chemicals, St. Louis, U. S. A.

2.3. Capillary electrophoresis

A Beckman P/ACE System 2100 capillary electrophoresis instrument with the cathode on the detection side was employed. Free zone capillary electrophoresis (FZCE) was used. All solutions were filtered through

a 0.22 μm pore membrane filter. Siderophore samples were analysed as iron (III) complexes. The siderophore-iron (III) complexes were prepared by adding a 10% excess of $FeCl_3$ in five steps at 30-min intervals and neutralising the solutions obtained with 0.1 M sodium hydroxide; the solutions were subsequently lyophilised. The capillary cassette was fitted with an uncoated fused-silica capillary, 57 cm in length (50 cm to the detector) $\times$ 50 μm I.D. Runs were made at a constant voltage of 20 kV. Pressure injection of the sample by 4 s was applied. The temperature of the capillary was maintained at 30°C and the separation effect was monitored at 214 nm. Analysis was carried out with 25 mM phosphate buffer, pH 12.5. All the samples investigated were dissolved in water. The electrophoretic data were acquired using System Gold software (Beckman).

3. Results and discussion

Capillary electrophoresis was applied as a fast method for siderophore identification in seawater samples. We identified ferrioxamine E and G in natural seawater samples (Fig. 4a). Peaks of ferrioxamine E and G with respective migration times of 4.2 and 4.9 min were identified in the subsurface seawater from the Gdańsk Deep. Co-injection of the natural sample with the siderophore mixture used as a standard showed excellent coverage of the ferrioxamine E and G peaks. A blank test did not show the presence of siderophores (data not shown).

Figures 4b–i show the electrophoregrams of seawater samples, including surface, subsurface and near-bottom seawater and sediment pore water from different regions of the Baltic Sea.

Ferrioxamine E (with migration times of 4.2 min) is the dominant siderophore, as it was found in all seawater and sediment pore water samples from different regions of the Baltic Sea, including the Gdańsk and Bornholm Deeps, the Słupsk Furrow and Puck Bay. Rhodotorulic acid (with migration times of $\sim$ 8 min) was identified in seawater samples from the euphotic zone (0–30 m) of Puck Bay and in sediment pore water from the Bornholm Deep and Puck Bay (Figs. 4e, 4h, 4i).

These results demonstrate that samples of seawater and sediment pore water from different regions of the Baltic Sea contain siderophores of the ferrioxamine family. The concentration of siderophores identified ranged from a few to a hundred nmol per dm^3 of seawater.

Neither ferrioxamines E and G nor rhodotorulic acid had been previously detected in the Baltic Sea. Iron chelators, the siderophores of the ferrioxamine family may be important in providing a form of iron in seawater readily assimilable by bacteria, cyanobacteria and algae.

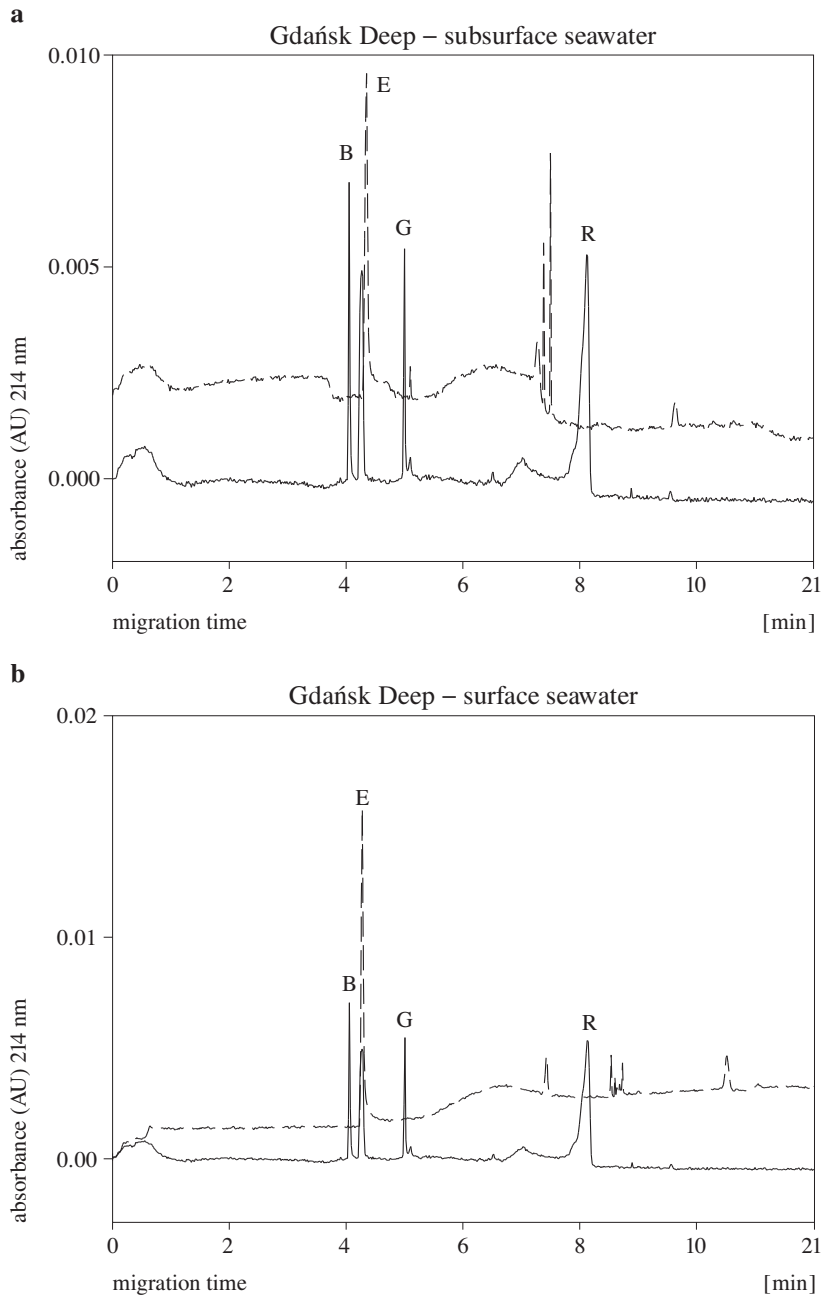
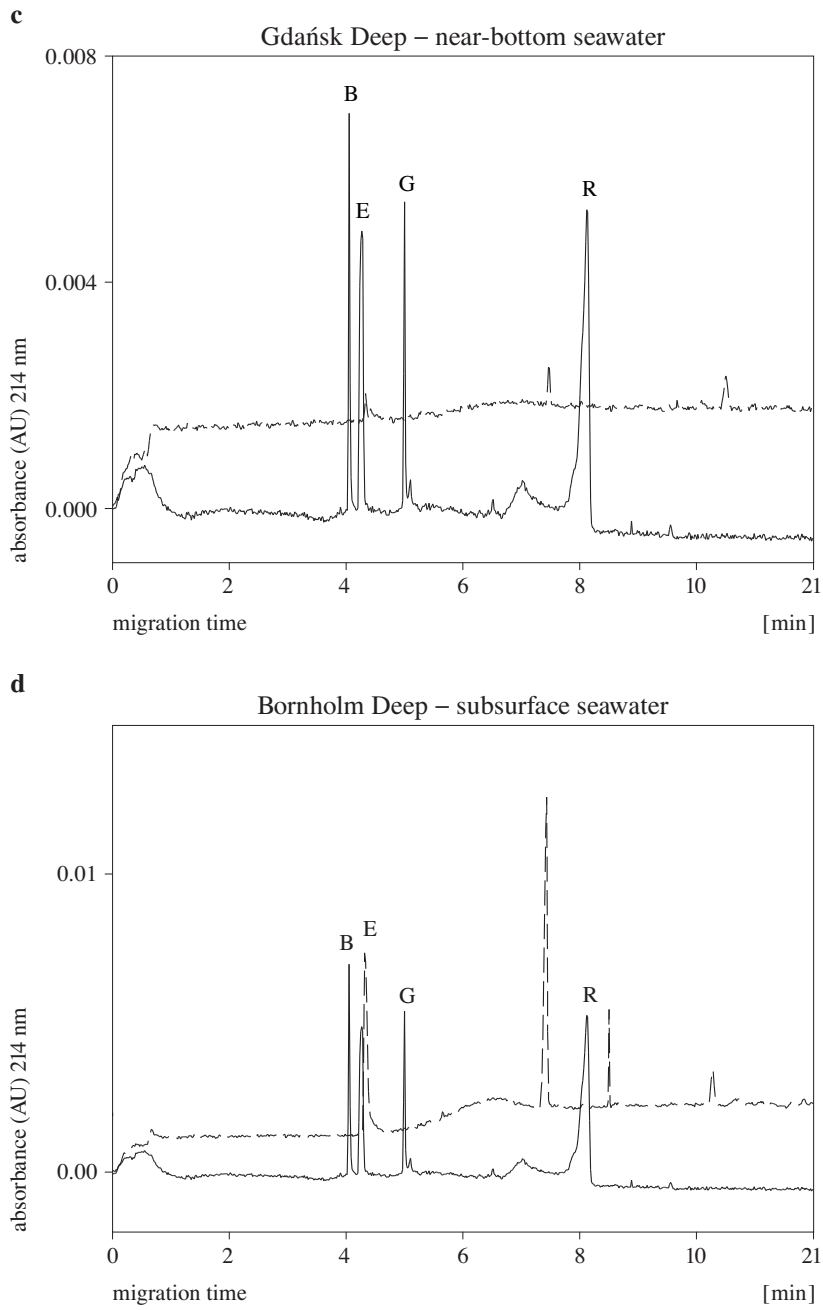


Fig. 4. Free zone capillary electrophoresis (FZCE) analysis of siderophores from sea and sediment pore water (a)–(i). Siderophore labels: ferrioxamine B (B), ferrioxamine G (G), ferrioxamine E (E), rhodotorulic acid (R). The siderophore standards concentration was 0.1 mg cm^{-3}. The lower solid lines represent the analysis of the siderophore standards



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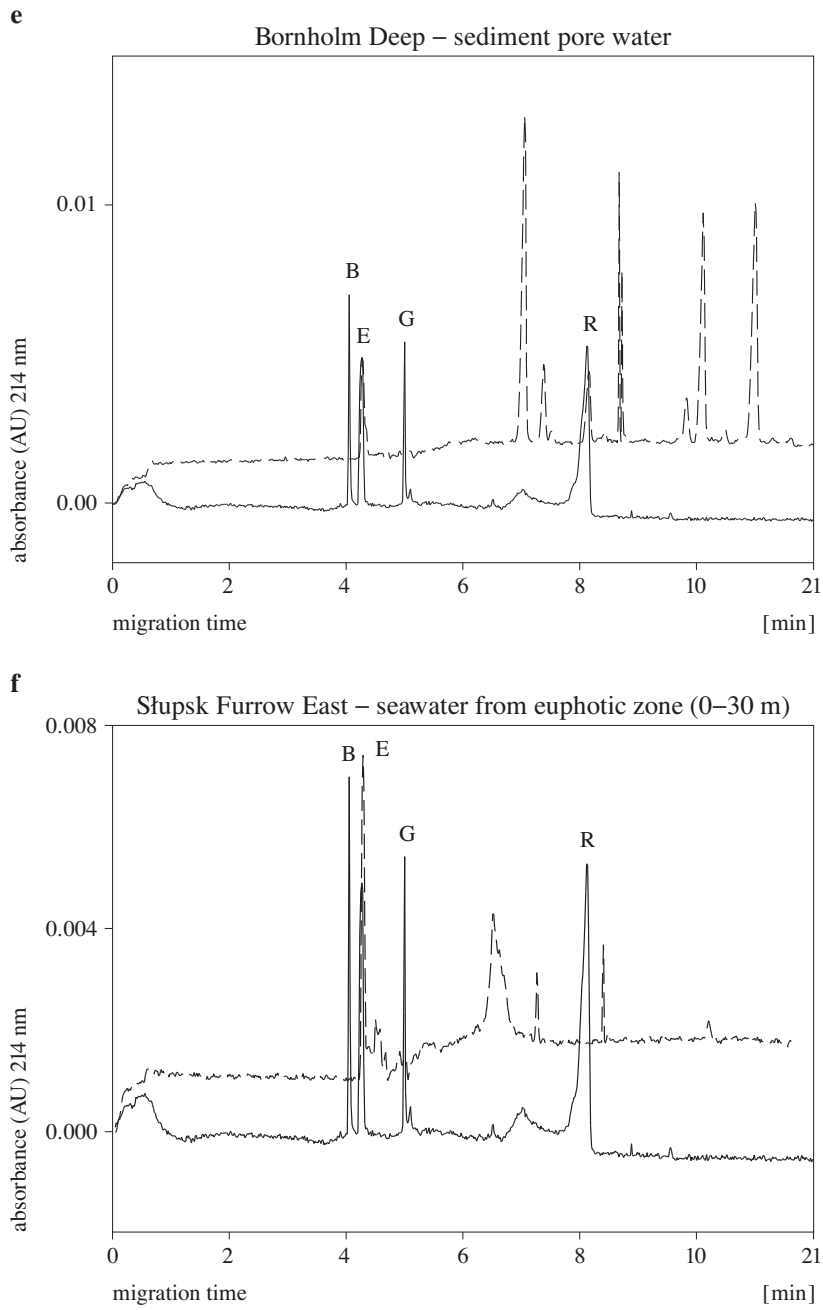

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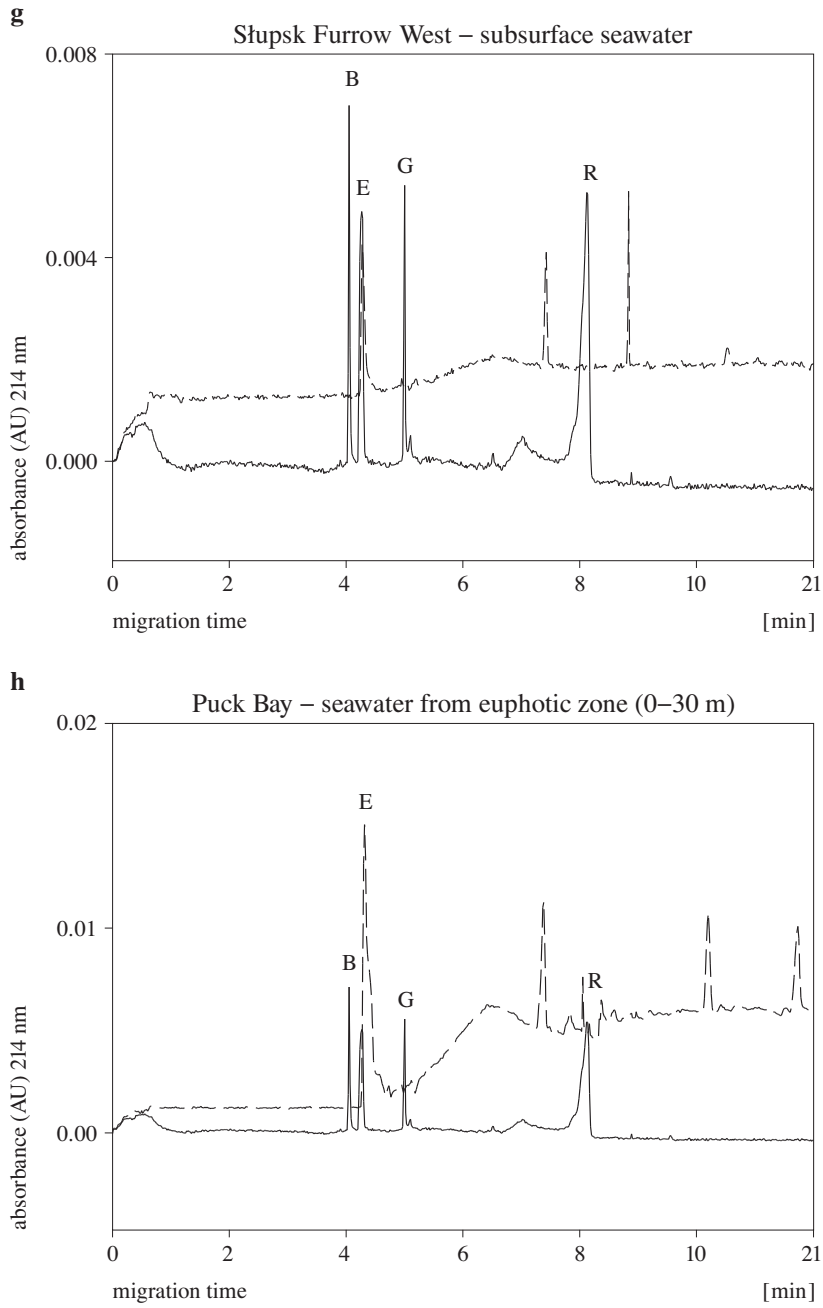

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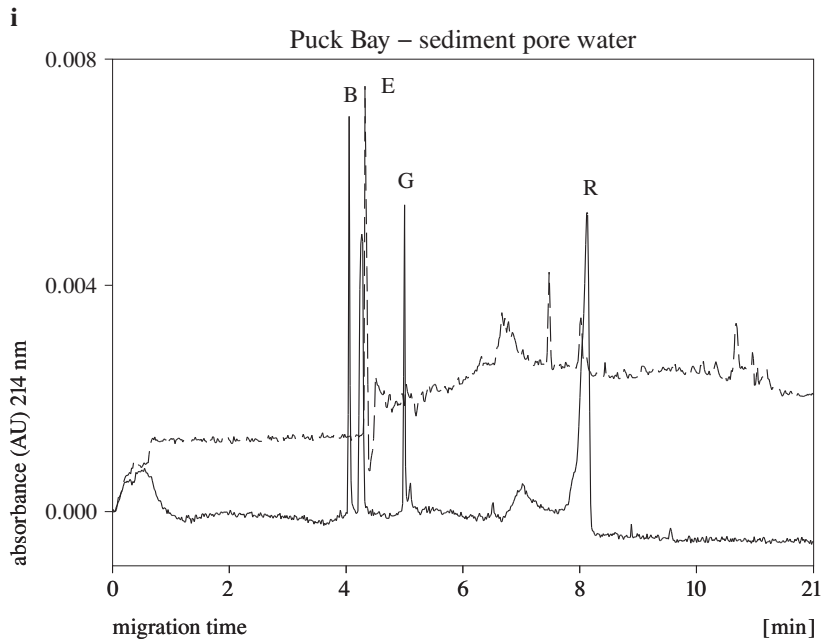

Fig. 4. (continued)

Additional peaks appeared on the electrophoregrams, but they did not correspond to any other standard siderophores analysed. In particular, the pronounced peaks with migration times of about 7.0–7.5; ∼8.5 and ∼10.0 min were not identified. Therefore, the presence of other siderophores containing hydroxamate or and phenolate/catecholate groups cannot be excluded.

These data suggest that ferrioxamine E and G and rhodotorulic acid in surface seawater from different regions of the Baltic Sea could be produced by cyanobacteria and bacterial cells rather than by algae. In subsurface seawater, near-bottom seawater and sediment pore water the same siderophores could be produced by bacteria, cyanobacteria and fungi. However, the chemical nature of the substances isolated suggests that bacteria are the dominant source of the compounds.

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