Aquatic Mammals 2000, **26.1,** 7–16

A cryo-scanning electron microscopic study of the skin surface of the pilot whale *Globicephala melas*

C. Baum[1], R. Stelzer[2], W. Meyer[3], D. Siebers[1] and L.-G. Fleischer[4]

[1]*Alfred Wegener Institute Foundation for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany*
[2]*Institute of Animal Ecology and Cell Biology, School of Veterinary Medicine, Bünteweg 17d, D-30559 Hannover, Germany*
[3]*Anatomical Institute, Department of Histology and Embryology, School of Veterinary Medicine, Bischofsholer Damm 15, D-30173 Hannover, Germany*
[4]*Institute of Food Technology, Technical University, Invalidenstraße 42, D-10115 Berlin, Germany*

Abstract

Surfaces of pilot whale (*Globicephala melas*) skin were studied by scanning electron microscopy with particular emphasis on surface condition and epibiontic marks of biofouling. To minimize artificial cross-linking of cell structures, dislocation of epibionts or dilution of salt crystals, untreated fresh skin samples were stored and examined by cryo-scanning electron microscopy at a controlled low temperature. The samples were compared with aldehyde-fixed skin of pilot whales.

The results obtained show that the surfaces of the fresh skin areas are covered with a smooth biofilm of alternating hydrophilic-hydrophobic sectors and exhibit negligibly low concentrations of epibiontic organisms or salt crystals. In contrast to the smooth biofilm observed in cryo-scanned samples, the aldehyde-fixed skin areas were covered with a more spongy layer of irregular structures, supposedly representing denatured remnants of the biofilm. We consider the biofilm to be the structural basis of skin smoothness and the primary site of epibiontic settlement.

Key words: pilot whale, *Globicephala melas*, skin, electron microscopy.

Introduction

Plants and animals living in the sea are exposed to epibiontic organisms, that attach to body surfaces and leave behind visible marks of biofouling (Wahl, 1989; Abarzua & Jakubowski, 1995). It is accepted generally that whales are frequently loaded with macrofouling organisms such as whale lice (*Cyamus* spp. and other related species) and barnacles, while on dolphins such marks of biofouling are usually limited to microfouling organisms such as diatoms and bacteria. As known from previous reports (Harrison & Thurley, 1972 and 1974; Ling, 1974; Haldiman *et al*., 1985; Liu Renjun *et al*., 1986; Pfeiffer, 1992; Pfeiffer & Jones, 1993; Pfeiffer & Rowntree, 1996), scanning electron microscopic images of chemically fixed skin samples of various cetacean species show typical marks of biofouling organisms only occasionally. Since these traces may be removed by organic solvents used for fixation and dehydration, we applied cryo-scanning electron microscopical techniques in this study, whereby organic solvents were avoided. We have designed an experimental approach to highlight new morphological features of the body surface of pilot whales and demonstrate a comparatively low degree of biofouling.

Materials and Methods

Skin samples were obtained from nine pilot whales (*Globicephala melas*) and one Atlantic white-sided dolphin (*Lagenorhynchus acutus*) entrapped in five separate events on the Faroe Islands (for further information see Bloch *et al*., 1993). From each animal five skin samples were taken from the flanks and the forehead (melon). Fresh and, by visual inspection, intact skin samples of the pilot whales were quench-frozen with liquid nitrogen and stored at -196°C until used.

A batch of tissue samples from both species was cut into 1.5×1.5 cm blocks and fixed at 4°C for 12 h using Schaffer's formol/ethanol mixture. The tissue blocks were kept in position with needles, dehydrated via increasing concentrations of ethanol (70–100%), air-dried with tetramethylsilane (Dey *et al*., 1989), and degassed over 24 h. We determined the deviation from the original size of the

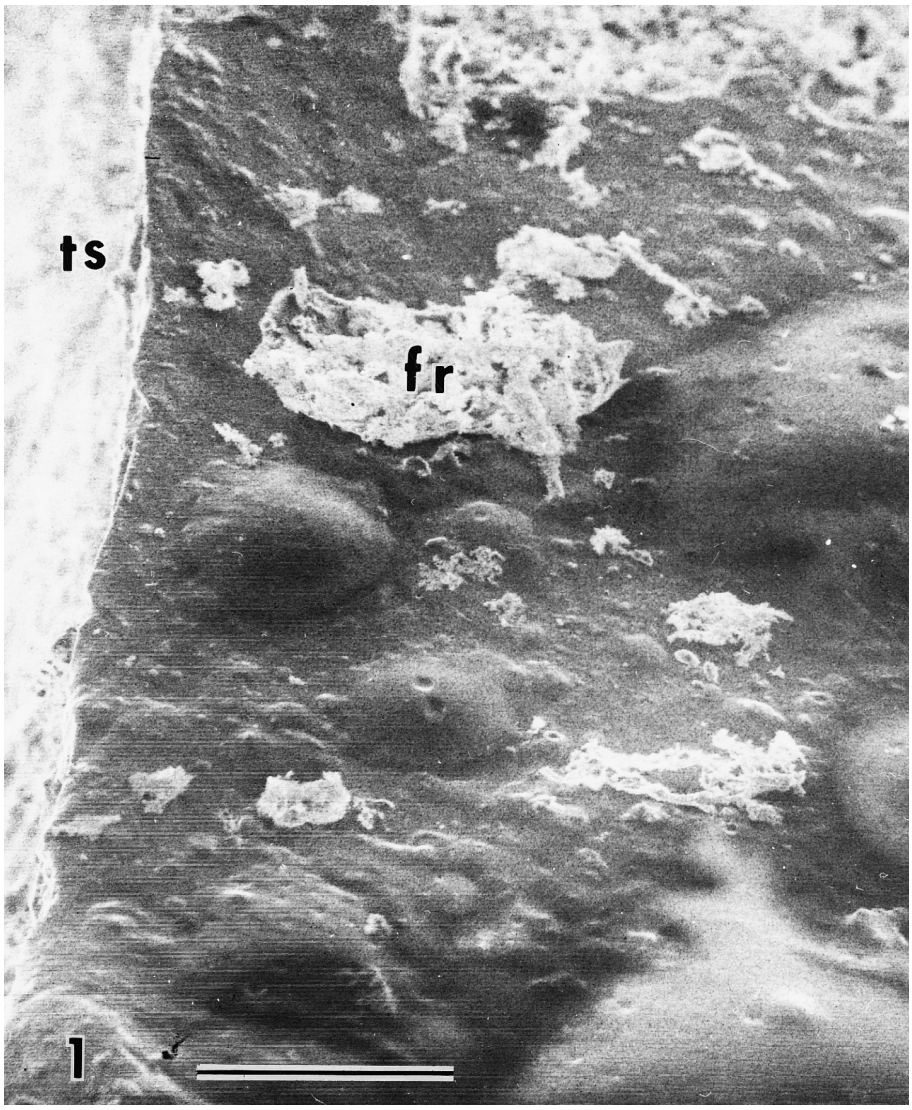
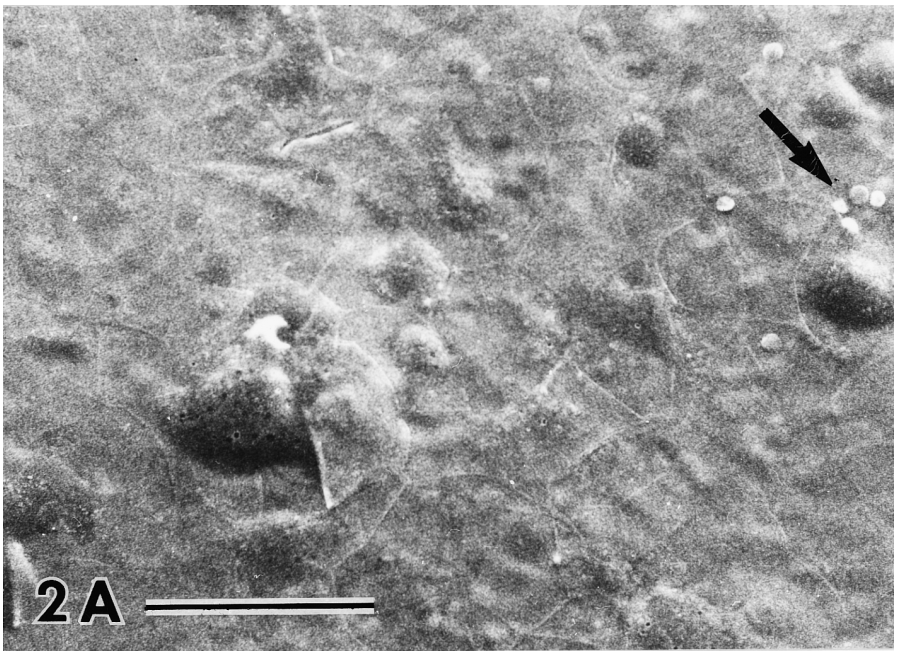


Figure 1. The skin surface of the pilot whale (*Globicephala melas*) displays plane and bulged areas. Fragments of the stratum spinosum (fr) upon the surface, after cryofracture. The tangential side of the block (ts) shows the stratum corneum and the underlying stratum spinosum. The sample was frozen-hydrated and non-coated. Scale bar = 100 μm.

blocks by direct measurement, and excluded blocks with a shrinkage of more than 10%.

For cryo-scanning electron microscopy, the quench-frozen skin samples were glued with Tissue Tek O.C.T. Compound (Miles Inc., USA) onto the surface of a copper-sample holder. Due to the melting point of Tissue Tek (approx. -10°C), it was necessary to warm the dermal face of the sample transiently up to -5 to -10°C and again cool it down to -196°C. The frozen samples were transferred to the cold-stage (-196°C) of the scanning electron microscope (SEM) ETEC Autoscan. The samples were viewed under high vacuum conditions (1.3×10^{-3} Pa), with an accelerating voltage of the electron beam of 10 kV and an emission current of 175 μA. To remove ice crystals from the surface, the samples were freeze-etched under electron optical control in the column of the SEM (within 20 s at -100°C and 1.3×10^{-3} Pa). Subsequently, without leaving the vacuum system of the



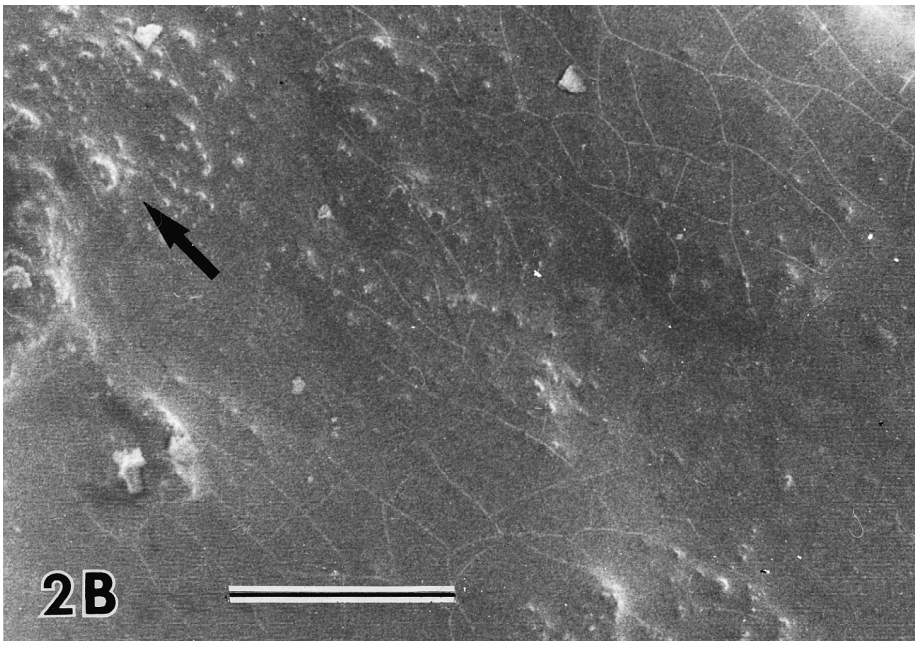


Figure 2. (A) Some diatoms (arrow) attached to the film covering the skin surface of the pilot whale *Globicephala melas*. (B) The biofilm enclosed salt crystals (arrow) clustered upon the surface of the pilot whale *Globicephala melas*. The samples were frozen-hydrated and non-coated. Scale bars = 100 μm.

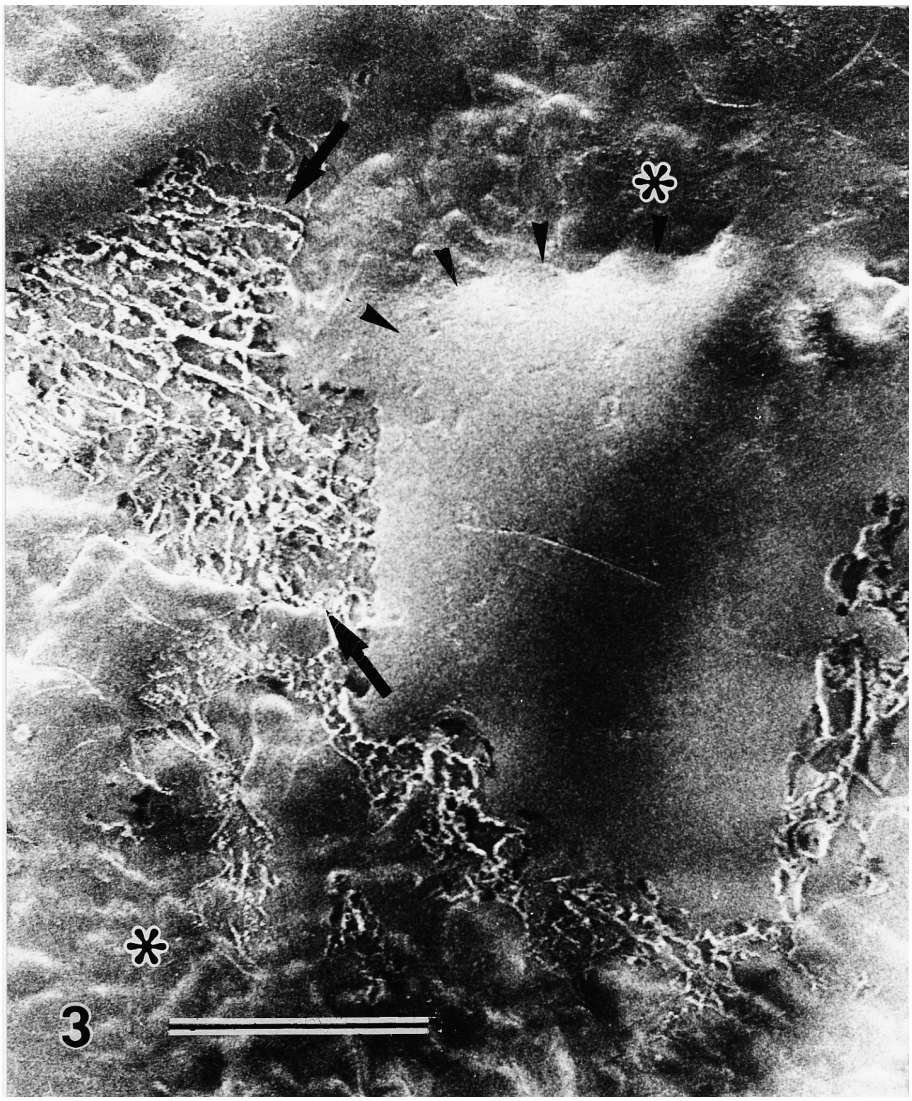


Figure 3. After careful freeze-drying, ice sublimated from hydrophilic components leaving behind alternately larger hydrophobic bulges (arrow head), larger hydrophilic spongy structures (arrow) and plane areas (asterisks) on the surface of the pilot whale (*Globicephala melas*). The sample was carefully freeze-etched and non-coated. Scale bar = 100 μm.

SEM, the surfaces of the samples were sputter-coated with aluminium/carbon under an argon atmosphere (8–10 Pa) in the prevacuum-chamber of the cryo-stage using a diode-sputter-coating system. Alternatively, some samples were freeze-dried for 12 h and sputter-coated with gold for 300 s under an argon atmosphere in a Hummer V sputter-coater (Technics).

The dried aldehyde-fixed samples were mounted on aluminium-sample holders with a conductive carbon paste (Leit-C). These skin samples were coated with carbon in the Edwards Coating System (E 306A) at 80 kV with five pulses of 0.5 s and thereafter, with gold for 90 s at 60 kV before examination in a Philips REM 515 scanning electron microscope at 10 kV.

Results

The uppermost layer of the stratum corneum of chemically untreated and frozen-hydrated pilot whale skin samples was smooth and covered with a film displaying plane and bulged areas (Fig. 1).

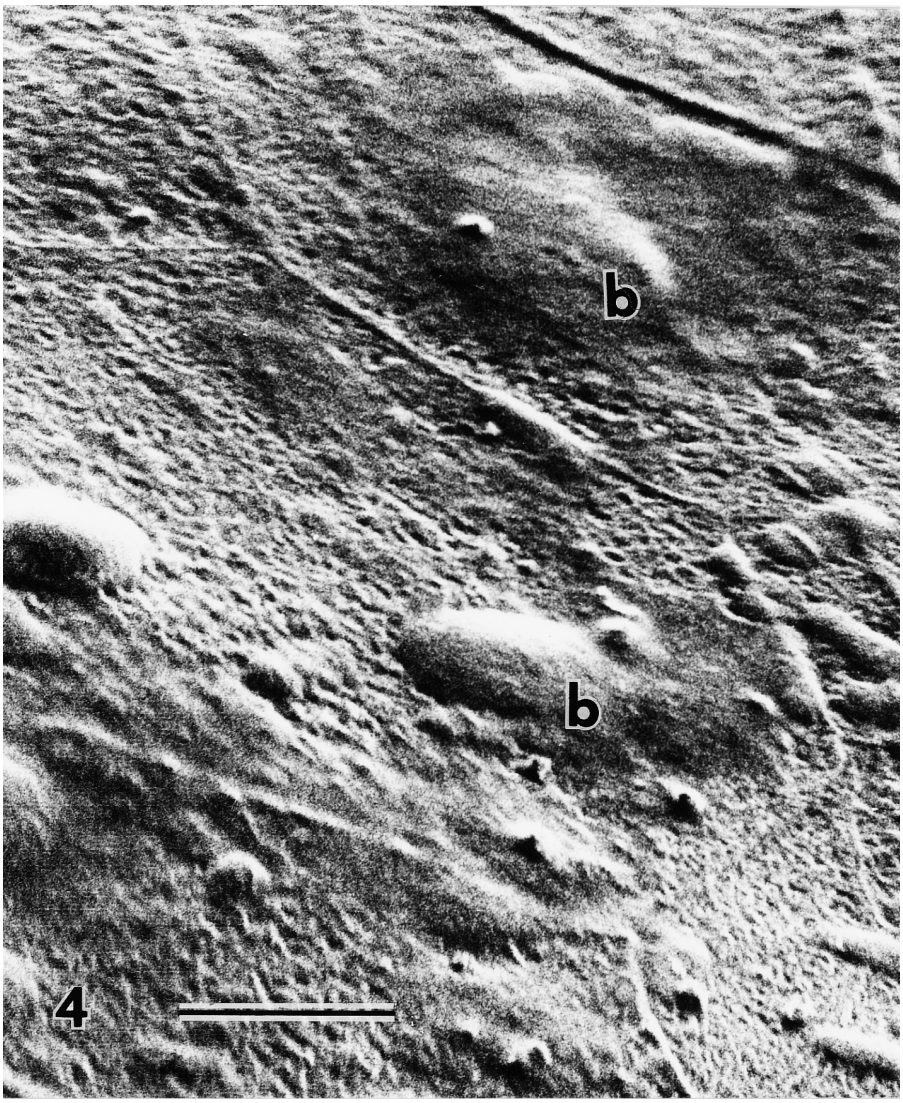


Figure 4. In higher magnification, the plane areas showed small hydrophobic bulges of about 1 to 5 μm in dia elevating from the surface of the pilot whale (*Globicephala melas*). These bulges (b) seem to correspond to the cavities of the nuclear remnants and lipid vacuoles. The sample was carefully freeze-etched and sputtered-coated with Al/C for 5 s. Scale bar = 20 μm.

The layer exhibited low densities of epibiontic organisms (Fig. 2A) such as diatoms or bacteria; inorganic deposits of salts or sand particles were extremely rare (Fig. 2B). Careful freeze-etching (Fig. 3) could split the film into alternating larger hydrophobic bulges, larger hydrophilic spongy structures and plane areas. Under higher magnification, the plane areas showed small hydrophobic bulges of about 1 to 5 μm in dia elevating from the smooth surface (Fig. 4). Compared with preliminary unpublished histological sections, these smaller bulges seem to correspond to the cavities of the nuclear remnants and the lipid vacuoles of the corneocytes seen in the sections.

At the beginning of the freeze-drying process, the formerly hydrated components of the plane film started to aggregate (Fig. 5). In aldehyde-fixed samples, however, irregular spongy structures and not aggregates covered the uppermost layer of the skin (Figs. 6A & 6B).

These irregular spongy structures were much less concentrated in aldehyde-fixed pilot whale skin

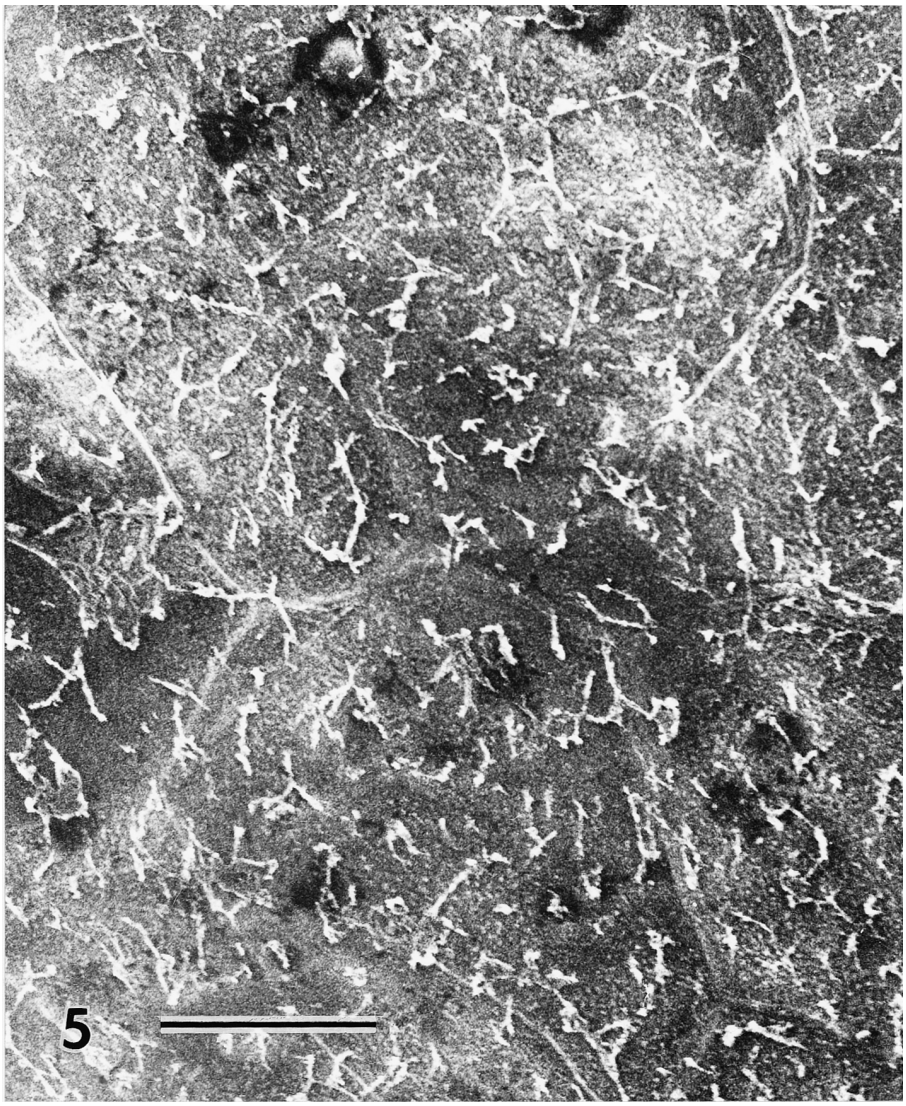


Figure 5. After a freeze-drying process within 20 s, hydrophilic components of the plane film areas started to aggregate on the skin surface of the pilot whale (*Globicephala melas*). The sample was freeze-dried and non-coated. Scale bar = 10 µm.

than in similarly treated specimens from an Atlantic white-sided dolphin *L. acutus* (Fig. 7). The empty cavities and channels seen in the aldehyde-fixed skin of *L. acutus* (Fig. 7) show some similarities to the hydrophobic bulges in frozen-hydrated skin samples of *G. melas* (Figs. 1 & 3). As argued above, the hydrophobic bulges may include lipids which were removed during the procedure of ethanolic dehydration of the skin. In Figure 7, these hydrophobic bulges thus appear as depressions of the skin surface representing the emptied cavities.

Discussion

Low temperature preservation used in this study enables us to largely avoid swelling or shrinking of tissue, dislocation of organisms and dilution of salt crystals. It was possible, for the first time, to demonstrate aggregation of hydrated components throughout a mild freeze-etching procedure. These surface aggregates showed similarities to those observed on aldehyde-fixed skin samples of various cetacean species (Harrison & Thurley, 1972 and

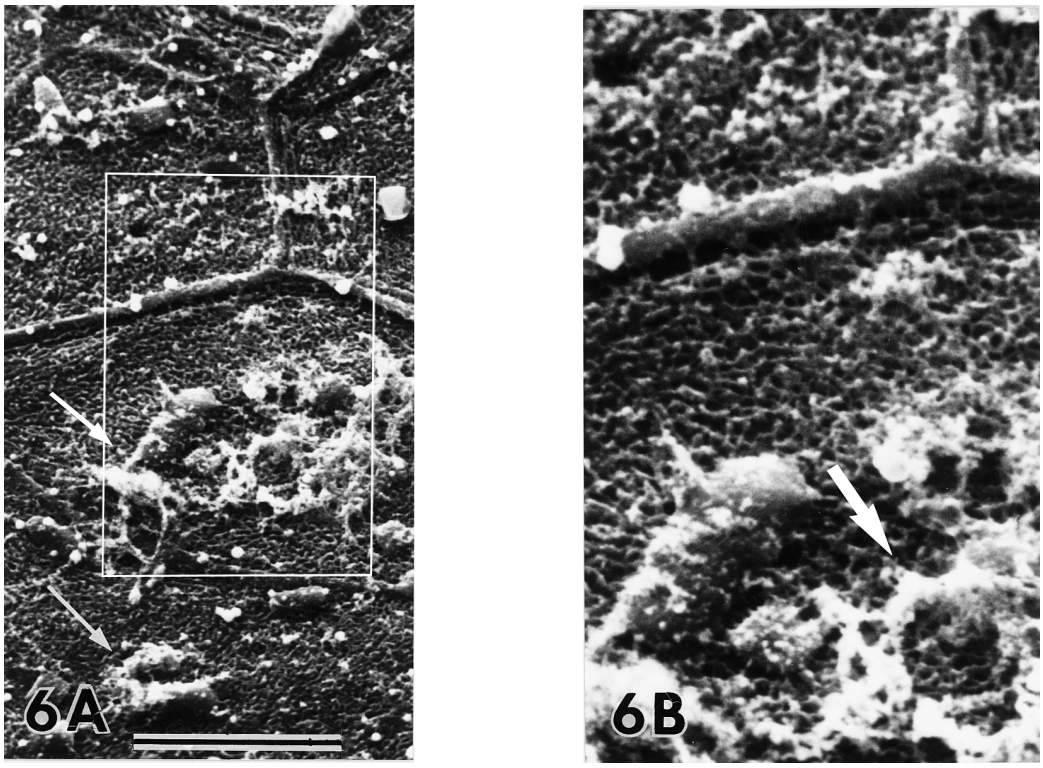


Figure 6. (A) In the aldehyde-fixed skin sample of the pilot whale (*Globicephala melas*), the surface was covered by irregular spongy structures (arrow) which share form patterns with freeze-dried skin samples (compare with Fig. 5). The sample was coated with carbon and gold. Scale bar = 15 μm. (B) Detail from square in Fig. 6A.

1974; Ling, 1974; Haldiman *et al*., 1985; Liu Renjun *et al*., 1986; Pfeiffer, 1992; Pfeiffer & Jones 1993; Pfeiffer & Rowntree, 1996). A new finding in pilot whales is that the uppermost layer of the stratum corneum of chemically untreated and frozen-hydrated skin samples is smooth and covered with a film of alternating hydrophobic-hydrophilic sectors. Since cetaceans lack skin glands (Spearman, 1972; Ling, 1974; Meyer *et al*., 1995), which in other mammals partially control epibiontic colonization, we conclude, in the case of the present skin samples of the pilot whale, that the biofilm could substitute for secretions released from cutaneous glands in other mammals. We consider that the biofilm covering the skin surface is analogous to fish slimes (Hoyt, 1975), forms the structural basis of skin smoothness and is the primary site of epibiontic settlement. The observation that the uppermost layer exhibits low concentrations of epibiontic organisms, such as copepods (Dierauf, 1990) fungi and bacteria (Dunn, 1990; Ushakowa, 1991), epizoic diatoms (Ling, 1974; Holmes, 1985; Denys, 1997), nematodes (Mariniello *et al*., 1994) and inorganic deposits of salts or sand particles (Vialé, 1984; Behrmann, 1996) indicates a strong self-cleaning ability of the biofilm. Because biofouling is a perpetual process (Lindner *et al*., 1992) self-cleaning mechanisms in dolphins must continuously be at work. Focussing on such mechanisms, we consider that self-cleaning abilities are based on several factors including desquamation (Brown *et al*., 1983; Hicks *et al*., 1985; St. Aubin *et al*., 1990), low surface tension effected by intercellular glycoconjugates (Gucinski & Baier, 1983; Baier *et al*., 1985) and gel formation of keratinocyte components, such as keratins and glycoconjugates. These factors might high efficiently cooperate in pilot whales as well as in bottlenose dolphins (Gol'din, 1994). But self-cleaning abilities might be negatively affected by discontinuous desquamation as in species with stronger periodical moulting tendencies (St. Aubin *et al*., 1990), or with annual migration behaviour to warmer water regions, such as in spermwhales and humpback whales, or in animals where the integument is permanently stressed mechanically, as, for example, by digging behaviour in grey whales.

The results obtained from the freeze-etching experiments showed the morphological correlate of

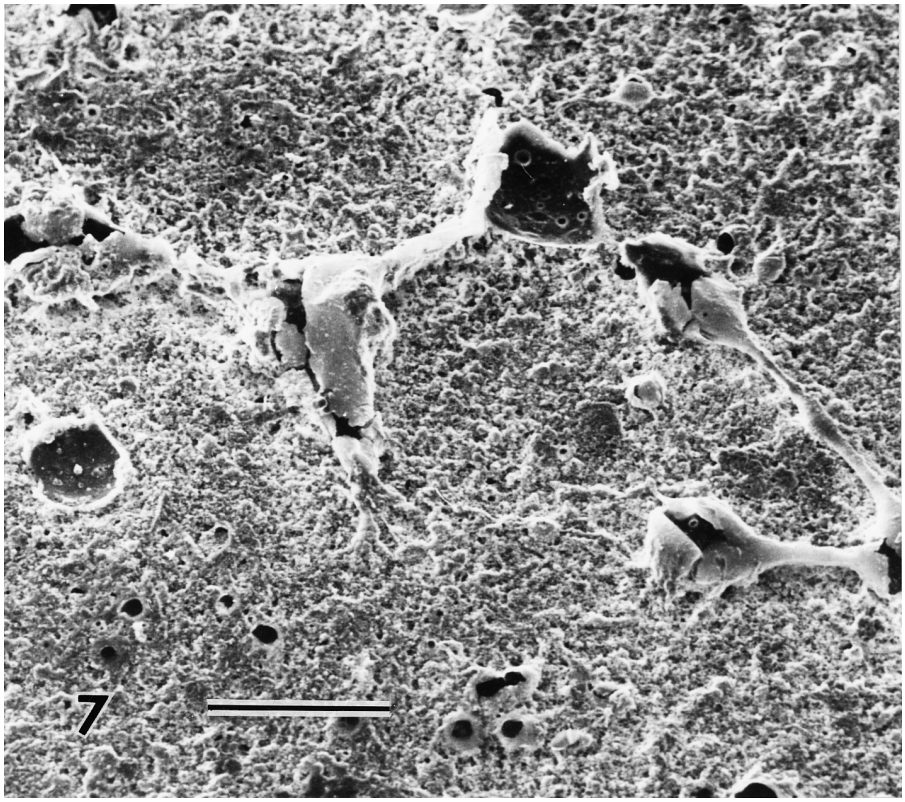


Figure 7. The aldehyde-fixed skin sample of the Atlantic white-sided dolphin (*Lagenorhynchus acutus*) displayed inclusions of lipids preserved as empty cavities, and channels after ethanolic lipid extraction, both more or less looking like the hydrophobic bulges covering the skin surface of the pilot whale (compare Figs 1 & 3). Scale bar = 100 μm.

low surface-energy effects. Since the smoothness of the biofilm is caused by a low surface tension (Gucinski & Baier, 1983; Gucinski *et al*., 1984) and the two discrete hydrophobic and hydrophilic fractions of the film (Fig. 3) did not emulsify, we argue that the two fractions exhibit the same comparatively low surface tension as the smooth surface of the frozen-hydrated samples. We conclude that the low surface tension of the skin exposed to seawater is affected by both hydrophilic glycoconjugates (Gucinski & Baier, 1983) and hydrophobic lipids, particularly non-polar acylglucosyl-ceramides (Menon *et al*., 1986).

In agreement with other delphinid species (Ling, 1974; Pfeiffer & Jones, 1993), the uppermost skin layers of the pilot whale displayed elevated surface areas and pockets (Fig. 6B) formed by the intensely interdigitating desmosomal junctions projecting from the keratinocytes to the skin surface as a network of microridges to guide the water flow and minimize the drag. But in the plane the microridges display the minor surface area, thus providing smaller effective attachable space for settlements in comparison to a completely plane plate. And the results only visible in the cryo-scanned samples indicated that the pockets between the microridges were filled with the biofilm, a good filling medium that, however, seems not strong enough for prolonged attachments. Within morphological limits, the grip of a biofilm within the microridges was predicted to a certain extent already by the macromolecular coating concept of Geraci *et al*. (1986).

We observed differences in height of the spongy irregular structures of aldehyde-fixed skin surfaces obtained from the pilot whale and the Atlantic white-sided dolphin (Fig. 7) allowing the assumption that the conditions of the skin-covering biofilm can be a determinant of the self-cleaning abilities of cetaceans, and a clue to the different patterns of biofouling in whales and dolphins.

Further comparative investigation on the boundary layer of the skin surface are planned for diverse cetacean species. Of particular interest in this

context are the biochemical and biophysical characteristics of the biofilm.

Acknowledgments

The authors thank Mrs. Dr. D. Bloch and Mr H. Gasadal, Museum of Natural History, Faroe Islands, Mrs. J. Zachariassen and Mr. Dr. A. Nørrevang, Department of Natural Sciences, University of the Faroe Islands, for their kind help with the field specimen collecting taken from legal harvest. Our appreciation also is expressed to Mrs. U. Bock and Mr. Dr. G. Kuhn, Department of Geology, Alfred Wegener Institute Foundation for Polar and Marine Research, and Mr. M. Gierth, Department of Animal Ecology and Cell Biology, School of Veterinary Medicine Hannover, for technical assistance in sample preparation. This study was supported by a grant of the Deutsche Forschungsgemeinschaft (ME 1755/ 1-1).

Literature Cited

Abarzua, S. & Jakubowski, S. (1995) Biotechnological investigation for prevention of biofouling. I. Biological and biochemical principles for the prevention of biofouling. *Marine Ecology Progress Series* **123,** 301–312.

Behrmann, G. (1996) Calcareous concretions in the skin of toothed whales (Odontoceti). *Archives of Fishery and Marine Research* **43,** 183–193.

Baier, R. E., Gucinski, H., Meenagham, M. A., Wirth, J. & Glantz. P. O. (1985) Biophysical studies of mucosal surface. In: S. A. Leach & T. Ericson (eds.) *Oral Interfacial Reactions of Bone, Soft Tissue and Salvia*, pp. 83–95. IRL Press Ltd., Oxford.

Bloch, D., Desportes, G., Mouritsen, R., Skaaning, S. & Stefansson, E. (1993) An introduction to studies of the ecology and status of the long-finned pilot whale (*Globicephala melas*) of the Faroe Islands, 1986–1988. *Report of the International Whaling Commission (Special Issue)*, **14,** 1–32.

Brown, W. R., Geraci, J. R., Hicks, B. D., St. Aubin, D. J. & Schroeder, J. P. (1983) Epidermal cell proliferation in the bottlenose dolphin *Tursiops truncatus*. *Canadian Journal of Zoology* **61,** 1587–1590.

Denys, L. (1997) Morphology and taxonomy of epizoic diatoms (*Epiphalaina* and *Tursiocola*) on a sperm whale (*Physeter marcocephalus*) stranded on the coast of Belgium. *Diatom Research* **1,** 1–18.

Dey, S., Basu Baul, T. S., Roy, B. & Dey, D. (1989) A new rapid method of air-drying for scanning electron microscopy using tetramethylsilane. *Journal of Microscopy* **156,** 259–261.

Dierauf, L. A. (1990) Marine mammals parasitology. In: L. A. Dierauf (ed.) *CRC Handbook of Marine Mammal Medicine: Health, Disease, and Rehabilitation*, pp. 89–96. CRC Press, Boca Raton, Florida.

Dunn, L. J. (1990) Bacterial and mycotic diseases of cetaceans and pinnipeds. In: L. A. Dierauf (ed.) *CRC Handbook of Marine Mammal Medicine: Health, Disease, and Rehabilitation*, pp. 73–87. CRC Press, Boca Raton, Florida.

Holmes, R. W. (1985) The morphology of diatoms epizoic an cetaceans and their transfer from *Cocconeis* to new genera, *Bennettela* and *Epipellis*. *British Phycological Journal* **20,** 43–57.

Geraci, J. R., St. Aubin, D. J. & Hicks, B. D. (1986) The epidermis of odonocetes: a view from within, In: M. M. Bryden & R. Harrison (eds.) *Research on Dolphins*, Part I, pp. 3–21. Clarendon Press, Oxford.

Gol'din, E. B. (1984) The black sea bottlenose dolphins (*Tursiops truncatus*) and overgrowing microalgae: some aspects of interrelations. In: *Proceedings of the First International Symposium on the Marine Mammals of the Black Sea, Istanbul, Turkey*, pp. 24–27.

Gucinski, H. & Baier, R. E. (1983) Surface properties of porpoises and killer whale skin *in vivo*. *American Zoologist* **23,** 959 (abstract).

Gucinski, H., Baier, R. E., Meyer, A. E., Fornalik, M. S. & King, R. W. (1984) Surface microlayer properties affecting drag phenomena in seawater. In: *Proceedings of 6th Marine Corrosion Congress, Athens, Greece*, pp. 1–24.

Haldiman, J. T., Henk, W. G., Henry, R. W., Alberet, T. F., Abdelbaki, Y. Z. & Duffield, D. W. (1985) Epidermal and papillary dermal characteristics of the bowhead whale (*Balaena mysticetus*). *Anatomical Record* **211,** 391–402.

Harrison, R. J. & Thurley, K. W. (1972) Fine structural features of delphinid epidermis. *Journal of Anatomy* **111,** 498–500.

Harrison, R. J. & Thurley, K. W. (1974) Structure of the epidermis in *Tursiops*, *Delphinus*, *Orcinus* and *Phocoena*. In: R. J. Harrison (ed.) *Functional Anatomy of Marine Mammals*, II/2, pp. 45–72. Academic Press, London.

Hicks, D. B., St. Aubin, D. J., Geraci, J. R. & Brown, W. R. (1985) Epidermal growth in the bottlenose dolphin, *Tursiops truncatus*. *Journal of Investigative Dermatology* **85,** 60–63.

Hoyt, J. W. (1975) Hydrodynamic drag reduction due to fish slimes. In: Y. Theodore, C. J. Brokaw & C. Brennen (eds.) *Swimming and Flying in Nature*, Vol. 2, pp. 653–672. Plenum Press, New York.

Lindner, E. (1992) A low surface free energy approach in the control of marine biofouling. *Aspects of Current Research of the US-Navy Biofouling Program* **6,** 193–205.

Ling, J. K. (1974) The integument of marine mammals. In: R. J. Harrison (ed.) *Functional Anatomy of Marine Mammals*, II/2, pp. 1–44. Academic Press, London.

Liu Renjun, Harrison, R. J. & Thurley, K. W. (1986) Characteristics of the skin of *Neophocoena phocaenoides* from Changjinang (Yangtse River), China. In: M. Bryden & R. J. Harrison (eds.) *Research on Dolphins*, pp. 23–31. Clarendon Press, Oxford.

Mariniello, L., Ceroni, S., Di-Cave, D. & Carlini, R. (1994) Metazoan parasites *of Stenella coeruleoalba*. *Parasitologia* **36,** 88.

Menon, G. K., Grayson, S., Brown, B. E. & Elias, P. M. (1986) Lipokeratinocytes of the epidermis of a cetacean (*Phocoena phocoena*). *Cell Tissue Research* **244,** 385–394.

Meyer, W., Neurand, K. & Klima, M. (1995) Prenatal development of the integument in Delphinidae (Cetecea: Odontoceti). *Journal of Morphology* **233,** 265–287.

Pfeiffer, C. J. (1992) Cellular structures of terminal baleen in various mysticete species. *Aquatic Mammals* **18,** 67–73.

Pfeiffer, C. J. & Jones, F. M. (1993) Epidermal lipid in several cetacean species: ultrastructural observations. *Anatomy and Embryology* **188,** 209–218.

Pfeiffer, C. J. & Rowntree, V. J. (1996) Epidermal ultrastructure of the southern right whale calf (*Eubalaena australis*). *Journal of Submicroscopic Cytology and Pathology* **28,** 277–286.

Spearman, R. I. C. (1972) The epidermal stratum corneum of the whale. *Journal of Anatomy* **113,** 373–381.

St. Aubin, D. J., Smith, T. G. & Geraci, J. R. (1990) Seasonal epidermal molt in beluga whales, *Delphinapterus leucas*. *Canadian Journal of Zoology* **68,** 359–367.

Ushakova, N. A. (1991) Microbiological examination of the skin and vaginal mucus of the Amazon freshwater dolphin (*Inia geoffrensis*) in relation to the physiological state of animal. *Proceedings of the Academy of Sciences of the U.S.S.R., Series Biology* **6,** 943–949.

Vialé, D. (1984) The salt-excretive function of the skin and changing coloration in cetacea. *Annals of the Institute of Oceanography, Paris* **60,** 87–93.

Wahl, M. (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Marine Ecology Progress Series* **58,** 175–189.