Marine Biosurfactants, III. Toxicity Testing with Marine Microorganisms and Comparison with Synthetic Surfactants

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Eight synthetic and nine biogenetic surfactants were tested on their toxicity. Because of their possible application as oil dispersants against oil slicks on sea, the test organisms used were marine microorganisms (mixed and pure cultures of bacteria, microalgae, and protozoa). Bacterial growth was hardly effected or stimulated, whilst that of algae and flagellates was reduced. All substances tested were biodegradaded in sea water. The bioluminescence of *Photobacter phosphoreum* (Microtox test) was the most sensitive test system used. A ranking shows that most biogenetic surfactants were less toxic than synthetic surfactants. No toxicity could be detected with the glucose-lipid GL, produced by the marine bacterium *Alcaligenes* sp. MM 1.

Introduction

During the last decade several surface active substances produced by microorganisms (biogenetic surfactants, biosurfactants) have been isolated and described [1–4]. Most of them are glycolipids composed of a hydrophilic sugar and of one or more lipophilic long-chain acids, *e.g.* corynomycolic acids.

An appropriate application of biosurfactants is the abatement of marine oil pollution. While the usage of synthetic oil dispersants is strongly limited by their toxicity, a better biodegradability and lower toxicity of biosurfactants could be expected because of their biogenetic origin. The first experimental investigations in this regard were made 1979–1981: The effect of crude oil and dispersed crude oil in tidal flat environments with the biosurfactant trehalose-dicorynomycolate (TL-2) and with the commercial dispersant Finasol OSR-5 was studied [5]. Less quantities of the oil penetrated into the sediment, was faster eliminated, and possessed a lower toxicity against *Corophium voluntator* (Amphipoda), after treatment with TL-2 compared with untreated oil or treated with OSR-5 [6]. However, data of a wider number of tested substances, test organisms, and test methods are still missing.

This paper deals with several toxicity testing series, in which numerous synthetic and biogenetic surfactants have been examinated, using several different test systems. The aim was to fill the gap described above and to give a ranking list basing on these data.

Materials and Methods

Surfactants

Chemically synthesized surfactants were EO 4,5 = nonylphenol-(ethylenoxide)$_{4,5}$-acetate (Hüls, Marl, F.R.G.), EO 9 = nonylphenol-(ethylenoxide)$_9$-acetate (Hüls, Marl, F.R.G.), TBS = tetrapropylene-benzene-sulfonate (Merck, Darmstadt, F.R.G.), CTAB = cetyltrimethyl-ammonium-bromide (Merck, Darmstadt, F.R.G.), DK 50 = sucrose-stearate, 30% monoester and 70% diester (Chemische Fabrik, Grünau, F.R.G.), DK 160 = sucrose-stearate, 70% monoester and 30% diester (Chemische Fabrik, Grünau, F.R.G.), Pril = a commercial cleaning surfactant (Böhme Chemie GmbH, Düsseldorf, F.R.G.), Corexit = the commercial oil dispergator Corexit 9527 (Esso, Hamburg, F.R.G.), and Finasol = the commercial oil dispersant Finasol OSR-5 (Fina GmbH, Frankfurt, F.R.G.). Biogene surfactants were TL-2 = tre-

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halose-dicorynomycolate and TL-4 = trehalose-tetraester (C_8, C_{10} – fatty acids, succinate), produced by *Rhodococcus erythropolis* DSM 43215, RL = rhamnose-lipid mixture, produced by *Pseudomonas sp.* DSM 2874, SS = sophorose-lipid-acid and SL = sophorose-lipid-lacton, produced by *Torulopsis bombicola* ATCC 22214, Suc = sucrose-lipid, produced by *Corynebacterium sp.* M 9b, GL = glucose-lipid, produced by *Alcaligenes sp.* MM 1, Emu = Emulsan, produced by the marine bacterium *Acinetobacter calcoaceticus* ATCC 31012, and LGP = sugar/protein-conjugate, produced by the marine bacterium SI-1 (strain classification in progress). Emu was obtained from Prof. Dr. D. L. Gutnik, Tel Aviv (Israel). All other biosurfactants were isolated and purified by the Institute of Biochemistry and Biotechnology, Braunschweig, F.R.G.). Detail information on the molecular structure of the tested surfactants is given in Fig. 1.

Bacterial growth inhibition

Acinetobacter calcoaceticus HO 1-N, *Photobacterium phosphoreum* NRRL B-11177, and *Serratia marinorubra* (subculture of the isolate from C. E. ZoBell) were obtained from the culture collection of Biologische Anstalt Helgoland, F.R.G. The bacteria were cultivated in a seawater medium (1 g/l bacto-pepton (Difco), 0.2 g/l bacto-yeast extract (Difco), 2% salinity), at 18 °C (dark, reciprocal shaker, 100 ml-flasks). The starting concentration was 10^4 cells/ml. The test medium was supplemented with 1, 10, or 100 mg/l (end concentration) surfactant. The bacterial growth was measured after 0, 0.25, 1, 2, 4, and 7 days with the pour plate method (medium: 5 g/l bacto-pepton (Difco), 1 g/l bacto-yeast extract (Difco), 10 mg/l $FePO_4 \times 4\, H_2O$, 15 g/l bacto-agar (Difco), 2% salinity). In each series the highest multiplication rate was documented.

Microalgae growth inhibition

Dunaliella tertiolecta (Chlorophyceae) was obtained from the culture collection of Biologische Anstalt Helgoland, F.R.G. The algae were cultivated in seawater medium (75 mg/l $NaNO_3$, 5 mg/l Na_2HPO_4, 3% salinity, 14 °C, 18 h daily illumination: 0.05 Einstein m^2/sec, 100 ml-flasks. The test medium was supplemented with 0, 1, 10, or 100 mg/l (end concentration) surfactant. The algae growth was measured using the direct counting method [7], and the maximum growth rate was documented.

Microflagellate growth inhibition

A mixed population of marine heterotrophic flagellates was enriched by inoculating 250 ml fresh collected seawater from the station "cable bouy, Helgoland" (German Bight, F.R.G.) with 250 ml seawater, supplemented with 1 g/l bacto-pepton (Difco). The mixture was cultured (18 °C, dark, reciprocal shaker). After 2 days a dense flagellate population has established through the propagation of the moderate growth of bacterial prey organisms. A dilution of this culture (now containing 100 fagellates per ml and a unknown number of saprophytic bacteria) was filled in 20 ml-bottles. The medium was prepared with 0–1000 mg/l surfactant in seawater (2% salinity) and 0.5 g/l pepton. The bottles were incubated at 18 °C in the dark on a shaker. The flagellate concentration was daily measured using a counting chamber. The test was judged negative (= the mass development of bacterivorous flagellates had been inhibited), if in one week not only 1 flagellate was detected in a single counting square (= the flagellate concentration is less than 10^5/ml), and the tested surfactant concentration was valued "toxic".

Biodegradation test

The biodegradation of surfactants was measured with the biochemical oxygen demand (BOD) – method in closed bottles. Fresh collected seawater from the station "cable-bouy, Helgoland" was supplemented with 1 mg/l surfactant, filled into 60 ml-bottles (under air exclusion), and incubated for 7 days at 18 °C in the dark. Every day one bottle was opened and the oxygen content measured. One serie of bottles without surfactant was tested as a control. The average daily surfactant degradation – measured as daily BOD – was documented.

Inhibition of bioluminescence

According to the standard method described previously [8], the surfactant concentration was measured, at which 50% of the bioluminescence of *Photobacterium phosphoreum* NRRL B-11177 is inhibited after 15 min treatment (effective concentration for 50% inhibition, EC_{50}).

Results

Inhibition of the growth of marine microorganisms. Bacterial growth was not generally inhibited by surfactants; even in a few cases stimulation occurred (Table I). For this reason it was impossible to calculate an EC-value from this data. In contrast to these results the multiplication of microalgae and microflagellates decreased in surfactant test series (Table II and III). Most synthetic surfactants were effective in lower concentrations than biosurfactants, which caused lower EC-values.

Table I. Influence of surfactants on the growth of marine bacteria; multiplication rate of the control series (= 100%): *A. calcoaceticus* = 9.3 d^{-1}, *P. phosphoreum* = 12.8 d^{-1}, *S. marinorubra* = 1.06 d^{-1} (NM = not measured).

Surfactant/	Concentration [mg/l]	Multiplication rate [%] *A. calcoaceticus*	*P. phosphoreum*	*S. marinorubra*
Control	0	100	100	100
TL-2	1	107	97	55
	10	118	87	92
	100	119	99	168
RL	1	126	101	60
	10	123	115	124
	100	111	110	432
EO 9	1	122	91	66
	10	140	109	85
	100	120	111	69
DK 50	1	106	96	NM
	10	82	95	NM
	100	66	109	NM

Table II. Growth inhibition of marine heterotrophic flagellates by surfactants; $EC_{fla\text{-}tox}$ = surfactant concentration, in which no mass development (over $10^5/cm^3$) occurred within 7 days.

Surfactant	$EC_{fla\text{-}tox}$ [mg/l]
Biosurfactants	
TL-4	>1000
TL-2	500–1000
GL	>1000
LGP	>1000
Suc	>1000
SL	100–500
SS	>1000
RL	25–50
Emu	>1000
Synthetic surfactants	
Finasol	13– 50
Corexit	50–100
Pril	25– 50
CTAB	3– 5
EO 4,5	15– 20
EO 9	60– 80
DK 50	>1000
DK 160	>1000

Table III. Inhibition of the growth of the marine microalgae *Dunaliella tertiolecta* (Chlorophyceae) by surfactants; multiplication rate of the control without surfactant (= 100%): 0.76 d^{-1}); negative multiplication means decreasing numbers of algae; EC_{50} = theoretical surfactant concentration of 50% inhibition.

Surfactant/	Concentration [mg/l]	Multiplication rate [%]	EC_{50} [mg/l]
Control	0	100	
GL	1	90	$\gg$3000
	10	91	
	100	88	
LGP	1	102	1585
	10	90	
	100	77	
SS	1	83	477
	10	71	
	100	78	
RL	1	96	20
	10	97	
	100	−59	
EO 9	1	86	500
	10	78	
	100	62	
DK 50	1	98	3000
	10	89	
	100	89	

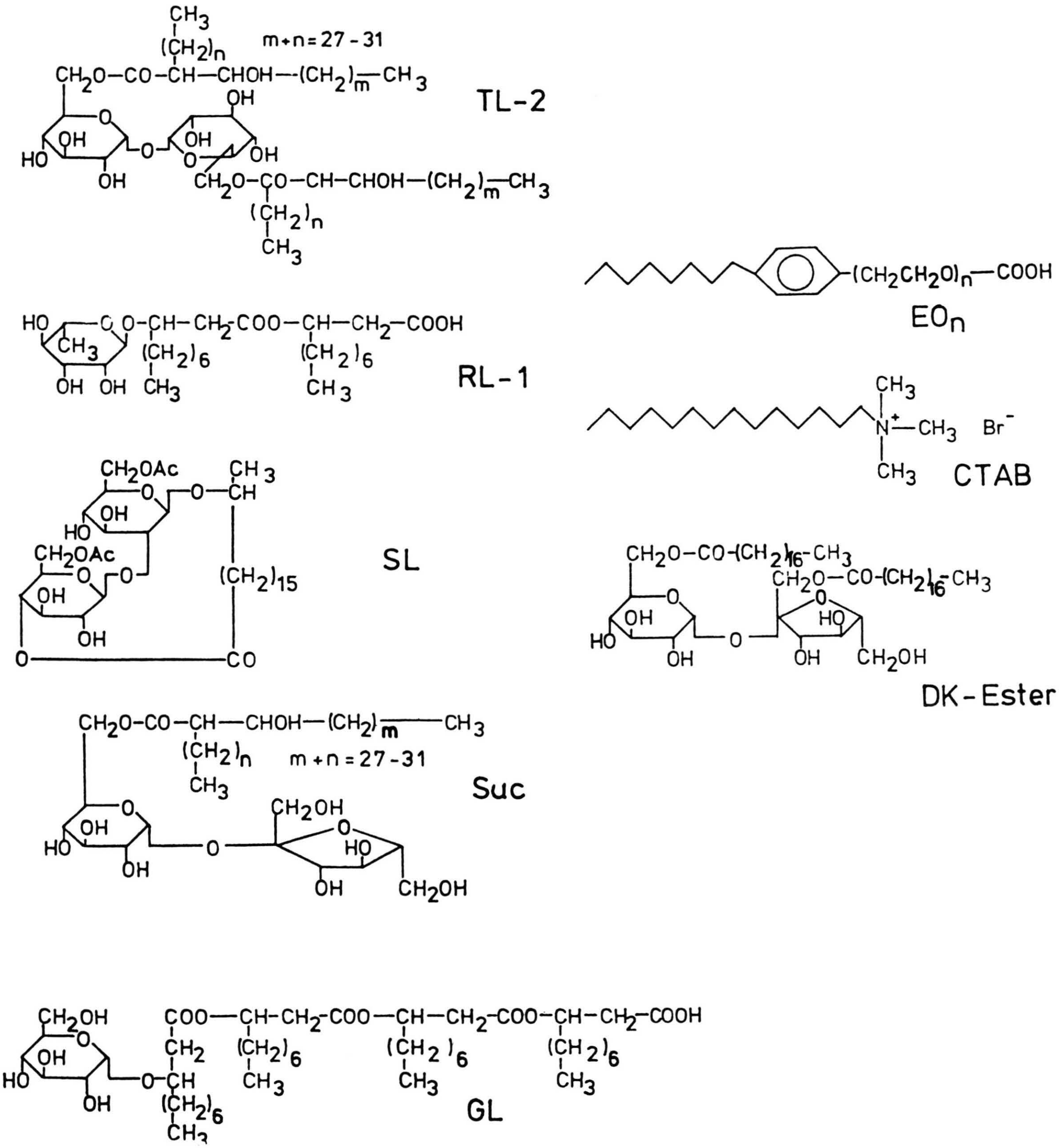

Fig. 1. Molecular structure of selected surfactants.

Biodegradation of surfactants. All surfactants tested were degraded by marine bacteria (Table IV). Biosurfactants were generally attacked faster than synthetic surfactants (exception: DK 50 and DK 160).

Bioluminescence inhibition. With the exception of GL all tested surfactants inhibited the luminescence of *P. phosphoreum* in varying amounts. Up to 100% reduction was observed with some of them, while others showed hardly any effect (Table V). Most EC-values of synthetic surfactants were higher than those of biosurfactants (exception: DK 50 and DK 160). Only SL and RL showed similar toxic effects. The missing small ef-

Table IV. Degradation of surfactants (1 mg/l) in seawater, measured as BOD per day.

Surfactant	Biodegradation (10^{-9} g O_2/l·d)
Biosurfactants	
TL-4	90
TL-2	108
GL	280
LGP	44
Suc	70
SS	142
SL	65
RL	190
Emu	130
Synthetic surfactants	
CTAB	35
EO4,5	10
EO9	40
DK 50	250
DK 160	260
Pril	44

Table V. Inhibition of the bioluminescence of *P. phosphoreum* by surfactants; EC_{50}, EC_{20} = effective concentration that inhibits 50 (20) % of luminescence; EC_{max} = maximal measured reduction of luminescence.

Surfactant	EC_{50} [mg/l]	EC_{20} [mg/l]	EC_{max} [%]
Biosurfactants			
TL-4	286	33	24
TL-2	49	7	43
GL		>3000	5
LGP	>3000	386	18
Suc	84	25	45
SS	141	12	54
SL	12	1	87
RL	50	6	100
Emu	202	10	50
Synthetic surfactants			
Finasol	7	1	100
Corexit	5	1	96
Pril	35	4	88
CTAB	0.5	0.3	100
EO4,5	79	38	45
EO9	78	7	57
DK 50	67	27	20
DK 160	334	88	17

ficiency of GL caused the impossibility to calculate an EC_{50}-value.

Discussion

In the forefield of a future application of biosurfactants in the sea, *e.g.* for the abatement of oil pollutions, the use of toxicity test systems dealing with marine organisms and test series with several biogenetic and synthetic products is usefull [9]. The first and (since now) only attempt in this direction was done by Henke [9], who measured the mortality of larvae of the brine shrimp *Artemia sp.* as parameter for surfactant toxicity. He found that the commercial oil dispersants Finasol OSR-5 and Corexit 9527 are about 1–2 magnitudes more toxic in this test system than several biogenetic glycolipids, *e.g.* trehalose-lipid, cellobiose-lipid, and sophorose-lipid. Unfortunately the disadvantage of the *Artemia*-method is the small ecological importance of the used test organism. Especially in the case of oil hazards the health of the ecosystem is rather influenced by the activity of bacterial degradation, microflagellate grazing, and microalgae photosynthesis. For that reason the application of microbial test organisms should be more useful.

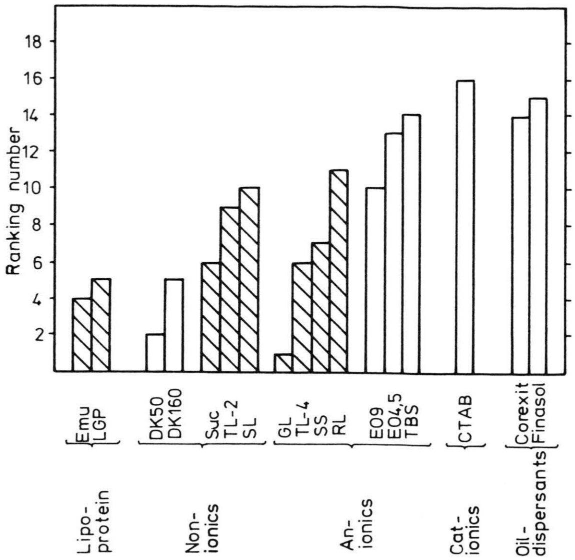

Fig. 2. Average ranking number of 17 surfactants concerning 4 test systems; a higher number stands for a greater toxicity; biosurfactants are shaded, synthetic surfactants are unshaded.

Up to now only a few data for the comparison and ranking of both synthetic and biogenetic surfactants are published. The mannosyl-erythritol-lipid Shizonellin B, the rhamnolipid R-2, and the lipopeptide Surfactin exihibited antimicrobial activity against several Gram-positive bacteria [10, 11], and the sophorose-lipids SL-1, 2, 3, and 4 from *Torulopsis bombicola* inhibited the growth of some Gram-positive organisms [12]. These results could hardly be taken in account for the following investigations, because Gram-positive bacteria are a negligible part of the marine population [13] and therefore were not used in our experiments. On the other hand, the greater sensitivity of Gram-positive bacteria compared with Gram-negative ones is well known [14].

Our findings document a generally greater sensitivity of marine eucaryotes than marine bacteria against surfactants. Similar results are known [15] for several other xenobiotics. The missing growth inhibition of bacteria could be the result of the biodegradability of surfactants, especially biosurfactants. Analogical results are known from *Pseudomonas aeruginosa,* that was not inhibited by biosurfactant SL but utilized it for growth [12, 16].

The better degradability of biosurfactants may be due to their specific molecular structure. While the synthetic EO-surfactants contain the difficult attackable aromatic benzene ring [17], the tested biosurfactants miss such an inert compound and should be totally mineralizable. The good oxidation of DK-surfactants is in conformity with this interpretation: DK-surfactants are synthetic glyco-lipids and of homological structure as the biogenetic glyco-lipids.

Greatest sensitivity against surfactants was found with the bioluminescence inhibition test: Less than 0.1 ppm CTAB inhibited *P. phosphoreum.* Already other authors [18, 19] have reported of the usage of this test system for toxicity screening of surface active substances. They found an increasing toxicity (measured as decreasing EC-value) with increasing lipophilicity of the surfactant. This confirms our observation, especially with TL-2/TL-4 and SS/SL, respectively. In each pair the more lipophilic partner was more toxic. (Other substances should not be compared with another, because of the missing homology of their hydrophilic molecular structure.)

The test systems used resulted in similar rankings of the tested substances, in which a high toxicity (high ranking number) stands for a low EC-value in growth or bioluminescence inhibition and slow biodegradation rate. It is possible to calcule an average ranking number (Fig. 2) as previously described [20]. The generally higher toxicity of synthetic products is significant. Only DK-surfactants behave different, due to their molecular structure similar with the biosurfactant Suc. It is described, that toxicity and ionogenic structure of the surfactants are related in that sense, that cationics are more toxic than anionics, and nonionics are the least toxic ones [14, 21]. This rule was obviously with the synthetic surfactants tested here. Biosurfactants miss this conformity; maybe, because their hydrophilic sugar-residue possess enough polar strength to mediate glycolipids an ionic-like character.

Finally, the small toxicity of GL is noteworthy. This "marine" surfactant missed nearly any response in growth inhibition tests and exhibites the fastest biodegradation of all tested substances. Nevertheless, we think it is too early to make its marine origin responsible for its missing toxicity against marine test organisms. GL has just been discovered [22] and further investigation should take place, before a special qualification of GL for an application in the marine environment could be stated.

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