Antibiotic resistance in marine neustonic and planktonic bacteria isolated from the Gdańsk Deep

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Abstract

This study deals with the antibiotic resistance of heterotrophic bacteria isolated from the surface and subsurface water of the Gdańsk Deep. The level of resistance of bacteria to various antibiotics differed considerably. As a rule, there were no significant differences in antibiotic resistance between neustonic and planktonic bacteria. Considerable diel fluctuations in bacterial antibiotic resistance were found. There were significant differences between pigmented and non-pigmented bacteria in their resistance to the tested antibiotics. Bacterial resistance to antibiotics was dependent on their chemical structure.

1. Introduction

To date, studies of bacterial antibiotic resistance have been focused mainly on clinical material, while little attention has been given to resistance of bacteria to antibiotics in natural environments (Kelch and Lee, 1978). This is why studies of the occurrence of antibiotic-resistant bacteria in aquatic ecosystems have recently been intensified (Niemi *et al.*, 1983; Hermansson *et al.*, 1987; Nair *et al.*, 1992; Silva and Hofer, 1995). A large number of bacteria, actinomycetales and algae occurring in aquatic ecosystems are capable of synthesising compounds of an antibiotic nature (Lemos *et al.*, 1985; Klein and Alexander, 1986). The concentration of these inhibitory substances in surface water could be *ca* 1 μg cm^{-3} (van Dijck and van de Voorde, 1976). The greatest amounts of antibiotics are produced by bacteria of the genera *Altromonas*, *Vibrio*, *Chromobacterium*,

Flavobacterium and *Pseudomonas* (Lemos *et al.*, 1985; Barja *et al.*, 1989; Dakhama *et al.*, 1993). Slowly but steadily, they synthesise and secrete into the water a number of antibiotic substances, namely phenazines, such as pyocyanine, 1-hydroxyphenazine, phenazine-1-carboxylic acids, oxychlororaphine, aeruginosin A and B, sulfazecin, isosulfazecin, and pyrrolnitrin (Williams and Vickers, 1986; Dakhama *et al.*, 1993). All of these compounds inhibit bacterial respiration and active transport of solutes by primarily interacting with the membrane and therefore disrupting the proton motive force (Baron *et al.*, 1989). They inhibit the growth of many marine bacteria, especially of the genera *Alcaligenes*, *Serratia*, *Proteus*, *Vibrio*, *Achromobacter*, *Bacillus* and *Aeromaonas* (Lemos *et al.*, 1985; Wu, 1993). Over 50% of marine actinomycetes synthesise antibiotics, mainly of the β-lactamase group, which inhibit bacterial growth (Okazaki and Okami, 1972; Williams and Vickers, 1986; Barcina *et al.*, 1987). Rasool and Wimpenny (1982) have calculated that *Streptomyces aureofaciens* should be able to produce enough tetracycline to prevent the growth of susceptible bacteria in a 10 μm radius around its hyphae. A lot of marine algae, mainly Chlorophycae, Rhodophycae and Phaeophyceae also produce substances of antibiotic character which inhibit the growth of Gram positive as well as Gram negative bacteria (Glombitza, 1969). Most often it is acrylic acid, in a minimal inhibitory concentration for bacteria of 0.030–38 mg cm^{-3}, that inhibits the activity of D-amino acid oxidase in bacteria (Sieburth, 1960).

The antibiotic resistance of bacteria is determined by the genes located in plasmids (Timoney *et al.*, 1978; Kobori *et al.*, 1984; Jeanthon *et al.*, 1991). There are four classic mechanisms of resistance specified by plasmids: inactivation, impermeability, bypass and altered target site (Davies and Smith, 1978). Also, intracellular binding now seems to be a valid mechanism for immobilising an inhibitor (Foster, 1983). According to Koch (1981), resistance can also be associated with the production of enzymes that modify and inactivate the antibiotic. Hermansson *et al.* (1987) drew attention to the fact that some strains of bacteria resistant to antibiotics do not contain any plasmids. However, the fact that the resistance of bacteria to an antibiotic is transferred by an extrachromosomal genetic factor (resistance factor R) seems more realistic (Kelch and Lee, 1978; Niewolak, 1984).

Although the problem of resistance of marine bacterial microflora to antibiotics is of a great significance in the ecology of these micro-organisms, it has been described in only a few papers to date (Tunstall and Gowland, 1974; Hermansson *et al.*, 1987; Nair *et al.*, 1992). This is why the aim of the present study was to determine the resistance of bacteria isolated from the southern Baltic Sea to antibiotics as an environmental selection factor.

2. Material and methods

Seawater samples were taken from the microlayer (ML), screen layer (SL) and subsurface layer (SUB) at 8 h intervals at a single station ($\phi = 55°01'$ N, $\lambda = 18°42'$ E) in the Gdańsk Deep. Falkowska (1996) describes the sampling site characteristics and the methods of seawater collection in detail.

In order to isolate neustonic bacteria (ML and SL layers) and planktonic bacteria (SUB layer), the samples collected were diluted with sterile seawater and inoculated by the spread method in five parallel replicates, on ZoBell 2216 E agar medium (ZB) prepared according to Rheinheimer (1977). Incubation was carried out at 20°C for 10 days. After that, bacterial colonies from each water layer were picked out and transferred to a semiliquid ZB medium. After purity control, the bacteria were stored at 4°C and subsequently used for further studies. In all, 60 strains of bacteria isolated from the microlayer, 79 strains from the screen layer, and 67 strains from the subsurface layer were studied.

The antibiotic resistance of neustonic and planktonic bacteria was determined by the single disc diffusion method (Niewolak, 1984; Lemos *et al.*, 1991; Silva and Hofer, 1995). Bacteria were multiplied on agar slants (ZB) at 20°C. After 72 h they were washed off the slants with 5 cm^3 of sterile seawater and adjusted to a turbidity of 4 on the MacFarland scale, which corresponds to 10^9 bacterial cells per 1 cm^3. Subsequently, 0.1 cm^3 of bacterial suspension prepared in this way was introduced into dissolved ZB medium, cooled to 40°C. After mixing, the sample was poured onto Petri dishes and dried in a drier at 37°C for 1 h. Paper discs impregnated with an antibiotic were then applied to the surface of the seeded medium. The blotting paper discs (ϕ 13 mm) used were manufactured by the Warsaw Serum and Vaccine Production Company and the Becton – Dickinson Company. The dishes were kept at 4°C for 1 h in order to allow antibiotic diffusion from the disc into the agar medium. The dishes were then incubated at 20°C for 24 h.

After incubation, the diameter (in mm) of the areas where bacterial growth was inhibited by the various antibiotics was measured. Bacteria were classified as antibiotic resistant only if they grew just as well as on plates prepared according to the manufacturer's instructions. The sixteen following antibiotics, with their concentrations given in parentheses, were used in the antibiograms: ampicillin (Ap, 10 μg), chloramphenicol (Cm, 30 μg), tetracycline (Tc, 30 μg), neomycin (Nm, 30 μg), streptomycin (Sm, 10 μg), novbiocin (Nb, 30 μg), kanamycin (Km, 30 μg), nalidixic acid (Nx, 30 μg), kindamycin (Kn, 2 μg), sulfamethoxazole (Sf, 23.75 μg), gentamycin (Gn, 10 μg), penicillin (Pn, 10 μg), doxycycline (Dc, 30 μg), trimethoprim (Tm, 1.25 μg), cloxacillin (Cl, 1 μg) and rifampicin (Rf, 10 μg).

The antibiotics were divided into six groups according to their chemical structure (β-lactams, aminoglycosides, tetracyclines, rifampicins, sulfonamides, others), (Foster, 1983; Kwiatkowski, 1992). The results were used to calculate the antibiotic resistance index (ARI) for bacteria according to the formula proposed by Jones *et al.* (1986) and Prieur (1989), modified by the authors.

3. Results

The results are presented in Fig. 1. These data show large differences in the level of bacterial resistance to different antibiotics. Over 90% of the bacterial microflora studied were resistant to trimethoprim, sulfamethazole, penicillin, cloxacillin, tetracycline, and kanamycin, while less than 10% of the strains were resistant to neomycin, gentamycin and rifampicin.

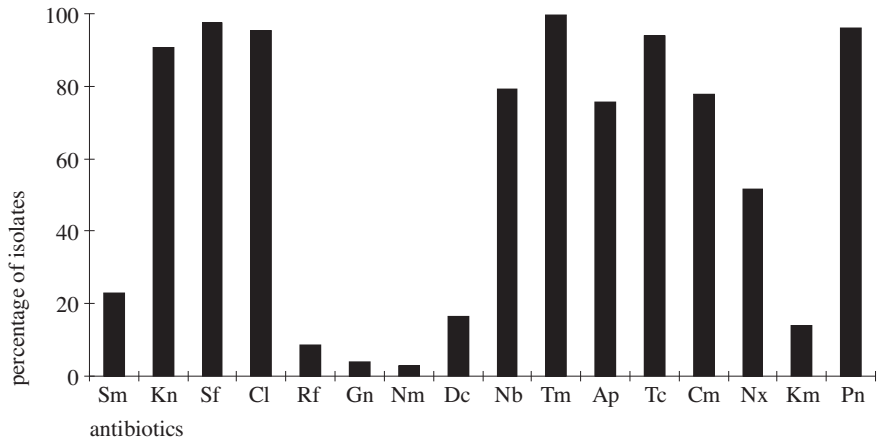

Fig. 1. Resistance of bacteria to different antibiotics among bacteria isolated from Gdańsk Deep area.
Explanations: Sm – streptomycin, Kn – kindamycin, Sf – sulfamethoxazole, Cl – cloxacillin, Rf – rifampicin, Gn – gentamycin, Nm – neomycin, Dc – doxycycline, Nb – novbiocin, Tm – trimethoprim, Ap – ampicillin, Tc – tetracycline, Cm – chloramphenicol, Nx – nalidixic acid, Km – kanamycin, Pn – penicillin

The data presented in Tab. 1 show that there were no significant differences between the bacteria isolated from different water layers in their resistance to the antibiotics used in this study. The value of the ARI in the three water layers studied was nearly identical (0.50–0.60), which indicates that this microflora has a similar potential antibiotic resistance. The only significant differences between the neustonic and planktonic bacteria occurred in their resistance to streptomycin. Over 50%

of planktonic bacteria (SUB) were resistant to this antibiotic, whereas the resistance of neustonic bacteria (ML and SL) was 5 to 10 times lower.

Table 1. Resistance to the antibiotics of bacteria isolated from different water layers (in percentage)

Antibiotic	Water layer		
	ML	SL	SUB
streptomycin	5.2	11.7	51.5
kindamycin	82.8	96.1	90.9
sulfamethoxazole	94.8	98.8	98.5
cloxacillin	94.8	94.8	97.0
rifampicin	5.2	7.8	12.1
gentamycin	1.8	3.9	6.1
neomycin	3.4	2.6	3.0
doxycycline	25.9	9.1	16.7
novbiocin	91.4	63.6	86.4
trimethoprim	100.0	98.7	100.0
ampicillin	70.7	77.9	77.3
tetracycline	98.3	92.2	92.4
chloramphenicol	75.9	80.5	75.8
nalidixic acid	67.2	48.1	42.4
kanamycin	20.7	6.5	16.7
penicillin	94.8	97.4	95.5
ARI	0.58	0.56	0.60

Explanations:
ML – microlayer,
SL – screen layer,
SUB – subsurface layer

The resistance of bacteria to various antibiotics fluctuated considerably in the diel cycle, however these changes did not display any distinct regularity (Fig. 2).

Significant differences in the antibiotic resistance between pigmented and non-pigmented bacteria were noted. Non-pigmented bacteria were more resistant than pigmented ones to 13 out of the 16 antibiotics tested (Fig. 3). Pigmented bacteria showed a higher level of resistance only to aminoglycoside antibiotics (gentamycin, neomycin, kanamycin).

The collection of strains was analysed for multiple antibiotic resistance (Fig. 4). Most of them were resistant to 8–11 antibiotics, but not a single strain was resistant to all 16. Multiantibiotic resistance was more common in planktonic than neustonic bacteria.

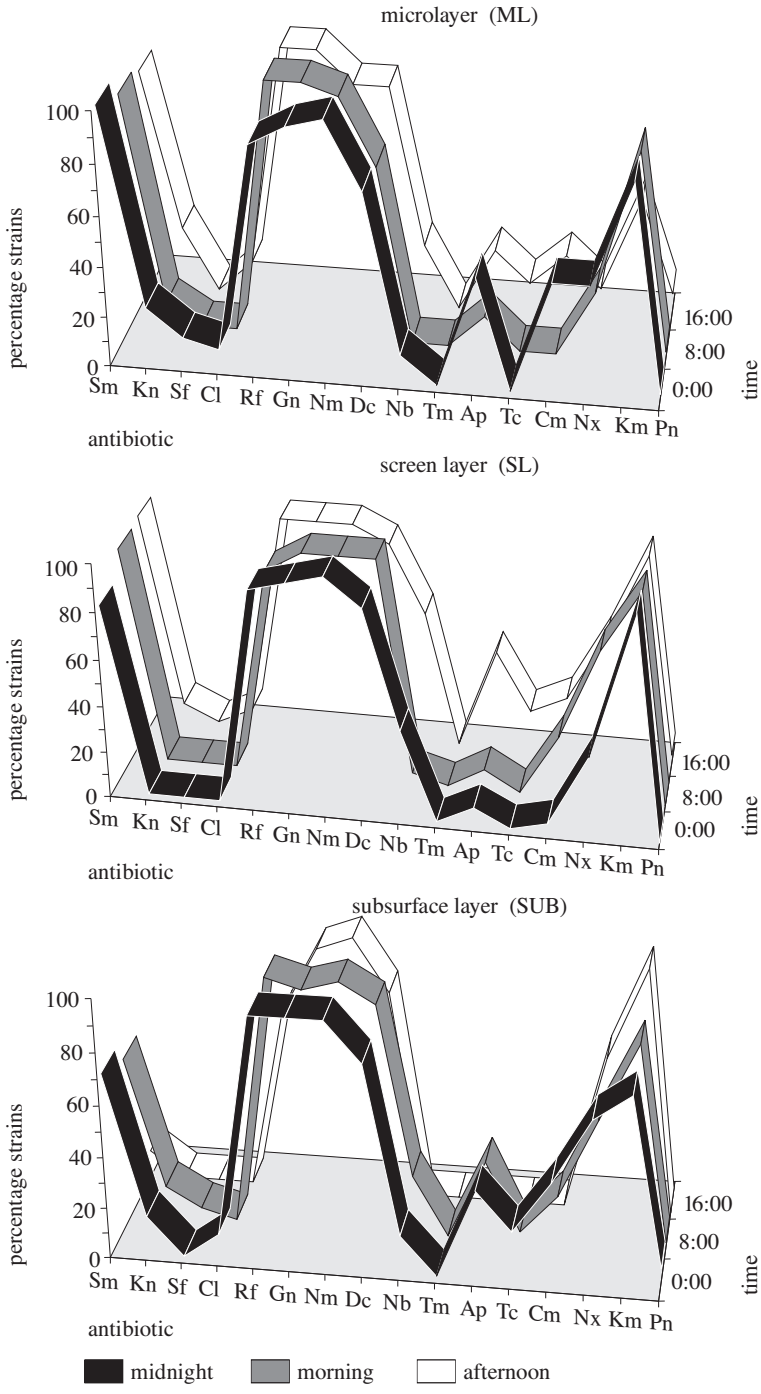

Fig. 2. Daily fluctuations in the resistance of bacteria to various antibiotics. Explanations as Fig. 1

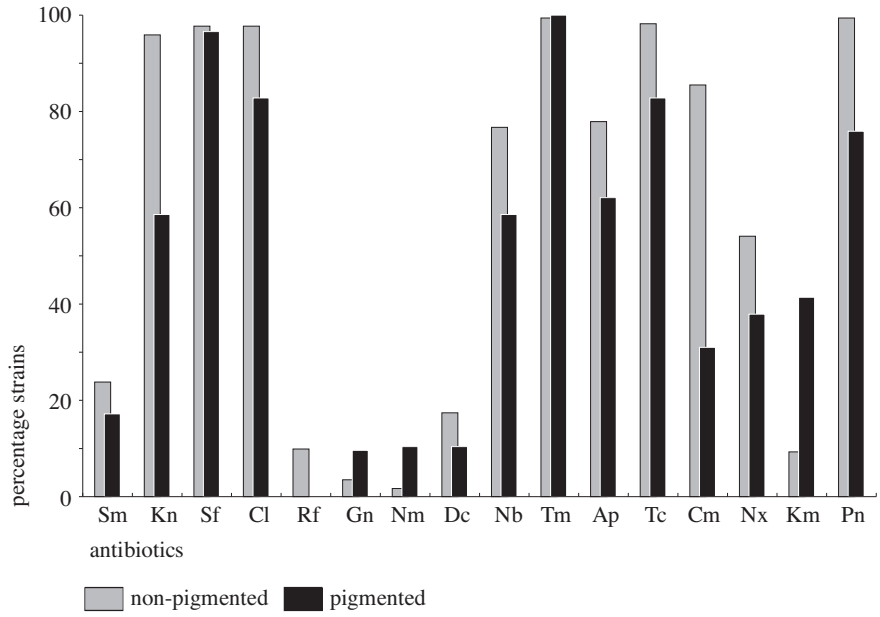

Fig. 3. Differential resistance of pigmented and non-pigmented marine bacteria to antibiotics. Explanations as Fig. 1

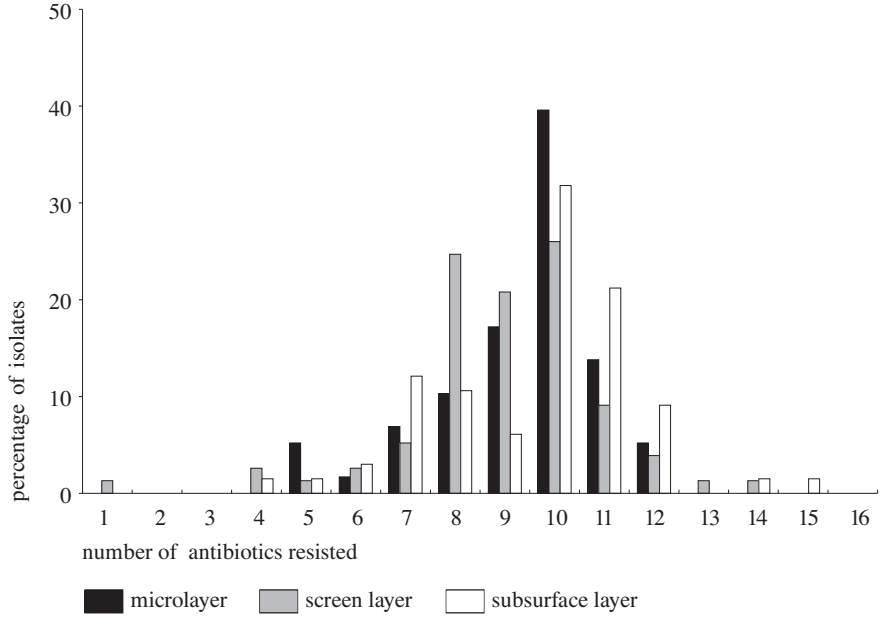

Fig. 4. Multiple-antibiotic resistant bacterial strains inhabiting the waters of the Gdańsk Deep

Tab. 2 sets out data on bacterial resistance to antibiotics in relation to their chemical structure. The strains examined were most susceptible to rifampicins and aminoglycosides, while a high percentage of the bacteria were resistant to the other groups of antibiotics, especially to the β-lactams.

Table 2. The resistance of bacteria to antibiotics with respect to their chemical structure (as a percentage)

Layer	AM	LA	TET	RYF	SUL	Others
ML	7.8	86.8	62.1	5.2	87.3	83.4
SL	6.2	90.0	50.6	7.8	81.8	80.1
SUB	19.3	89.9	58.3	12.1	80.3	84.4
Average	11.1	88.9	57.0	8.4	83.1	82.6

Explanations:
AM – aminoglicosides (neomycin, streptomycin, kanamycin, gentamycin),
LA – β-lactams (penicillin, ampicillin, cloxacillin),
TET – tetracyclines (tetracycline, doxycycline),
RYF – rifampicins (rifampicin),
SUL – sulfonamides (chloramphenicol, kindamycin, novbiocin),
Others – (sulfamethoxazole, nalidixic acid, trimethoprim)

4. Discussion

The function of antibiotic substances in natural ecosystems is one of the most controversial topics in the field of microbial ecology. While some authors argue that antibiotics are waste products excreted by microorganisms, others propose that antibiotic production is a deliberate behaviour on the part of certain micro-organisms (Williams and Vickers, 1986; Lemos *et al.*, 1991). Although our knowledge about the ecological significance of antibiotics is still incomplete, many microbiologists are convinced that they play an important role in the interaction between organisms inhabiting different ecological niches.

The survival of bacteria in water bodies is dependent on the level of their tolerance to physical, chemical and biological stress. The occurrence of antibiotic substances produced mainly by other bacteria and algae is one such stress factor (Olssen *et al.*, 1964; Klein and Alexander, 1986; Dakhama *et al.*, 1993). Hence, the level of resistance to those inhibitors determines the survival of marine bacteria.

The level of resistance of marine bacteria isolated from the waters of the Gdańsk Deep to various antibiotics differed considerably. The strains were

most resistant to trimethoprim, sulfamethazole, penicillin, cloxacillin and tetracycline while they were most susceptible to neomycin, gentamycin and rifampicin. In other water bodies, a high level of resistance to penicillin and tetracycline and a high level of susceptibility to neomycin and gentamycin was also noted by Strzelczyk *et al.* (1971), Niewolak (1984), Jeanthon *et al.* (1991), Lemos *et al.* (1991), Silva and Hofer (1995). Niemi *et al.* (1983) determined that bacteria inhabiting the northern Baltic Sea were much more resistant to ampicillin than to streptomycin. The same results were obtained by the authors of the present paper for the southern part of the Baltic Sea.

The studies carried out by Hermansson *et al.* (1987) and Jones *et al.* (1991) show that bacteria inhabiting the surface layer are much more resistant to antibiotics than those isolated from deeper water layers. By contrast, the results obtained in the present study do not show any significant differences in antibiotic resistance between neustonic and planktonic bacteria. According to Kelch and Lee (1978) and Niemi *et al.* (1983), the antibiotic resistance of bacteria depends on their taxonomic position rather than their origin.

Hermansson *et al.* (1987) and Nair *et al.* (1992) determined that pigmented bacteria were more resistant to antibiotics than non-pigmented ones. In this study, contrary results were obtained.

Bacteria inhabiting many water bodies are resistant to only a small number of antibiotics (Niemi *et al.*, 1983; Nair *et al.*, 1992; Silva and Hofer, 1995). In the present study, however, the majority of bacterial strains inhabiting the waters of the Gdańsk Deep were found to be characterised by multiantibiotic resistance. This indicates that marine bacteria are perfectly capable of detoxicating those antibacterial factors.

The results of numerous studies indicate that bacterial resistance to antibiotics is dependent on their chemical structure (Foster, 1983; Hermansson *et al.*, 1987; Nair *et al.*, 1992). This relationship is also confirmed in our study, as neustonic and planktonic bacteria isolated from the Gdańsk Deep were most susceptible to aminoglycosides and rifampicins. Aminoglycoside antibiotics interfere with bacterial ribosomal translation, *i.e.* with protein synthesis, which is responsible for their inhibitory effect. As a defence, bacteria synthesise three enzymes – phosphotransferase, acetyltransferase, and adenyltransferase – which inactivate this group of antibiotics (Shannon and Phillips, 1982). The data obtained by the authors indicate that the bacteria studied here are able to synthesise these enzymes only to a limited extent, since such a high percentage of those organisms were sensitive to aminoglycosides.

Zemelmann *et al.* (1980) have indicated the high resistance of marine bacteria to β-lactam antibiotics. In the present study, neustonic and planktonic bacteria were also found to have the greatest resistance to this group of antibiotics. The resistance of bacteria to β-lactam antibiotics lies in their ability to synthesise three enzymes, of which β-lactamase and acylase limit the permeability of the cytoplasmatic membrane to those antibiotics, whereas penicillinase transforms those compounds into antibiotically inactive penicilloic acid (Legakis *et al.*, 1978).

This study has proven that antibiotics are a significant selection factor in the marine environment. Hence, it appears worthwhile to carry out further studies in order to determine the role of antibiotic substances in controlling the populations of marine bacteria.

5. Conclusion

The results presented above allow the following conclusions to be drawn:

- Bacteria isolated from the Gdańsk Deep display large differences in the level of resistance to different antibiotics.
- There are no significant differences between the bacteria isolated from different water layers in their resistance to the antibiotics used in this study.
- The resistance of bacteria to various antibiotics fluctuated considerably during the diel cycle.
- Multiantibiotic resistance occurred more commonly in planktonic than neustonic bacteria.
- Significant differences in antibiotic resistance between pigmented and non-pigmented bacteria were noted.
- Bacterial resistance to antibiotics was dependent on their chemical structure.

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