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# Impacts of Cultivation of Marine Diatoms on the Associated Bacterial Community▽

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**The composition of bacterial communities associated with four diatom species was monitored during isolation and cultivation of algal cells. Strong shifts in the associated communities, linked with an increase in the numbers of phylotypes belonging to members of the *Gammaproteobacteria*, were observed during cultivation.**

A close linkage of bacterio- and phytoplankton has been assumed in the past, leading to the "phycosphere" concept (1). Strong associations of certain bacterial morphotypes and phytoplankton cells in cultures and natural seawater have been shown previously (12). Nevertheless, identification of these bacteria could not be achieved. Hence, microalgal batch cultures were examined with easy access to microbial biomass for molecular analysis (8, 11, 17, 19).

These experiments are usually conducted with microalgae from culture collections which had been isolated long ago and cultured for years. Three null hypotheses are generally possible: long-term cultures led to selection of bacterial populations because of (i) specific association, (ii) nonspecific cocultivation, or (iii) cocultivation with genetic variants of algal cells over time.

We investigated the bacterial community composition during initial steps of isolation and subsequent cultivation of diatom species to assess possible effects due to cultivation. To our knowledge this is the first time that cultivation shifts have been described in detail for diatom-associated bacteria.

**Algal cultures.** Single diatom cells were isolated as described previously (17). These were *Guinardia delicatula* (Cleve) Hasle, *Pseudonitzschia pungens* Grunow, *Thalassiosira rotula* Meunier, and *Skeletonema costatum* (Greville) Cleve, dominating in the water of Helgoland Roads, Helgoland, Germany (22). Microalgae were incubated in f/2 medium (9) at 16°C with 12-h-light–12-h-dark cycling at 20.0 μmol photons $m^{-2}$ $s^{-1}$. After 2 months, single cells were grown to clonal cultures. Afterwards, every month, fresh medium was inoculated with cells of the respective cultures (dilution factor, 0.02). Samples were taken after 2, 4, and 12 months in the early stationary phase of algal growth (steps 1, 3, and 11). Samples of the in situ community retrieved at the date of isolation were used to cover the original bacterial community (16). A 50-ml volume of each sample was filtered through successive membrane filters (17).

**Community analyses.** Nucleic acid extraction, amplification of intergenic spacers, ribosomal intergenic spacer analysis (RISA), amplification of 16S rRNA gene fragments, and denaturing gradient gel electrophoresis (DGGE) were carried out as described previously (16). Sequencing of excised DGGE bands and analyses of sequence data were performed as already described (4, 14, 17). These resulted in sequences of approximately 500 bp. Sequences

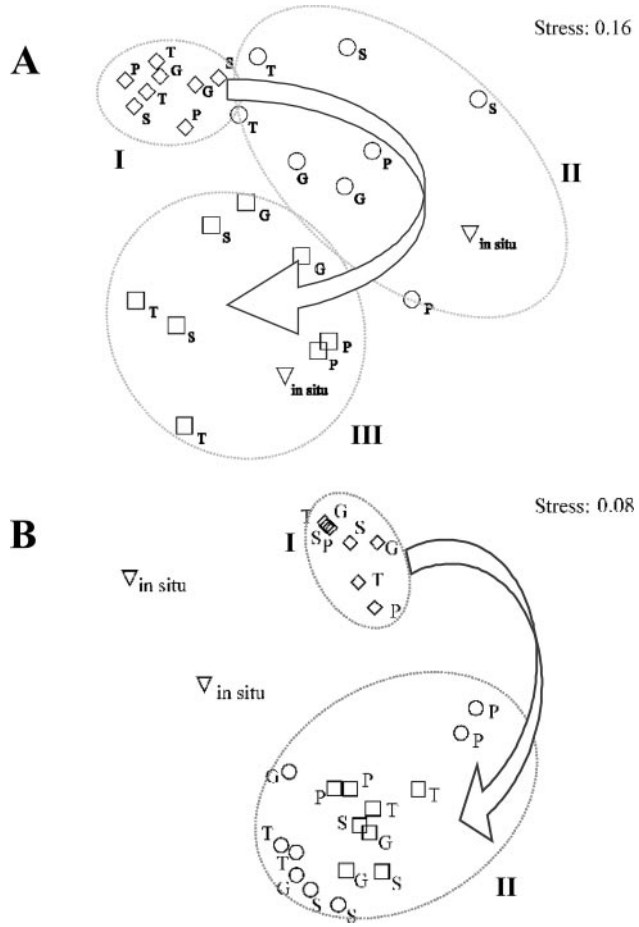


FIG. 1. MDS plots based on Bray-Curtis similarities of community fingerprints of different microalgal cultures (G, *Guinardia delicatula*; T, *Thalassiosira rotula*; P, *Pseudonitzschia pungens*; S, *Skeletonema costatum*), two sample fractions, and four sampling dates. Cultivation steps are indicated as follows: ▽, in situ; ◇, step 1; ○, step 3; and □, step 11. The arrows indicate the development of the communities. Circles display groups of samples. (A) MDS plot of RISA fingerprints. (B) MDS plot of DGGE fingerprints.

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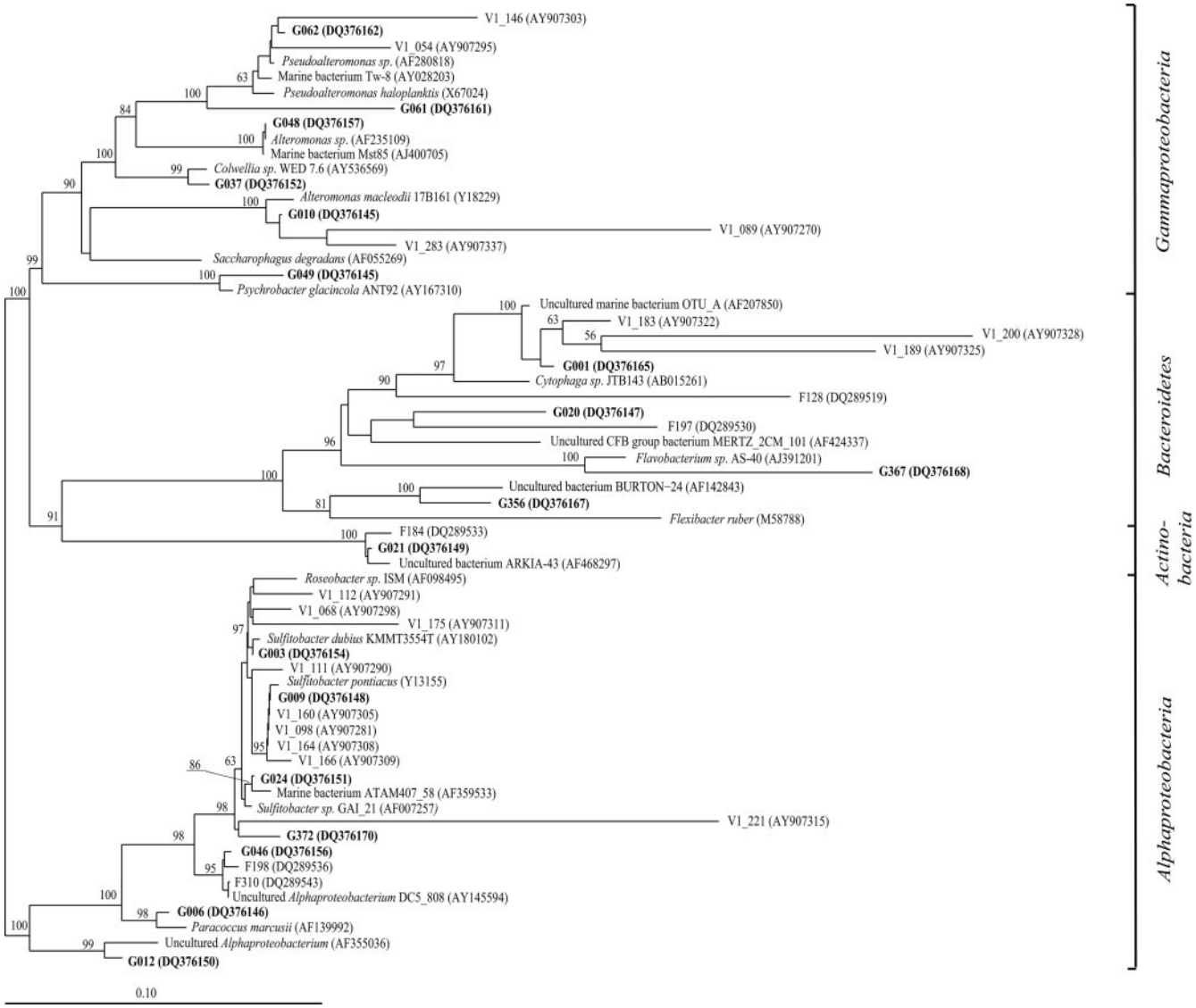


FIG. 2. Phylogenetic tree of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, and *Bacteroidetes* spp. GenBank accession numbers are given in parentheses. Bootstrap values above 50% are displayed.

within an ARB database related to sequences retrieved in our study and relatives found by BLAST (http://www.ncbi.nlm.nih.gov) displaying more than 1,300 nucleotides were used to calculate phylogenetic trees (17).

RISA and DGGE profiles were analyzed using BioNumerics 4.5 (Applied Maths BVBA, Belgium). Multivariate analysis of fingerprints was performed using the subroutines ANOSIM and MDS of PRIMER 5 software (3) as described previously (17). The null hypothesis was that no difference between associated communities of different cultivation steps exists. A sample statistic ($R$; significance level of 0.1%) displayed the degree of separation (17).

**Community shifts.** The MDS plot of RISA fingerprints (Fig. 1A) displayed a development of associated communities within three steps. Communities associated with freshly isolated microalgae could be grouped together (group I). These were not similar to the in situ community. The communities displayed a shift after 4 and 12 months (group II, pairwise test, $R = 0.561$; group III). The communities within group III were strongly separated from the communities associated with freshly isolated microalgae (Fig. 1A) (pairwise test, $R = 0.835$). General separation of communities was also reflected by ANOSIM analysis (global $R = 0.636$). A similar development was observed by MDS analysis of DGGE fingerprints (Fig. 1B), displaying a separation of communities (global $R = 0.771$). A shift occurred after 2 to 4 months (pairwise test, $R = 0.787$), resulting in more similar communities after 4 to 12 months (group II, pairwise test, $R = 0.434$). The associated communities in step 1 are completely separated from the communities in step 11 (pairwise test, $R = 1.0$).

**Phylogeny.** Most sequences were related to those of *Alphaproteobacteria* and *Gammaproteobacteria* spp. (44% and 31%; Fig. 2). The results revealed matches with 98 to 100% similarity to bacterial 16S rRNA gene sequences. A total of 19% of the

phylotypes belonged to *Bacteroidetes* spp., and 6% (two phylotypes) affiliated to *Actinobacteria* spp. A neighbor-joining tree revealed that sequences belonging to *Alphaproteobacteria* spp. clustered mainly with *Sulfitobacter* spp., and sequences related to those of *Gammaproteobacteria* spp. were mainly assigned to *Alteromonadales* spp. (Fig. 2). Some phylotypes are already found in microalgal cultures retrieved from phytoplankton of Helgoland Roads (G001, G009, G062) (17). Phylotypes G021 and G046 clustered with sequences retrieved from the in situ sample (16).

Several authors identified members of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* as being associated with algal cells (2, 7, 8, 10, 11, 12, 17, 19, 21). Members of these bacterial groups might play an important part in the interaction of bacteria and microalgae.

However, in most if not all previous studies concerning phycosphere communities, members of *Actinobacteria* were not detected. Therefore, it is not clear whether this phylotype truly represents bacterium-phytoplankton associations.

**Succession of phylotypes.** A total of 18 phylotypes were identified at different positions on DGGE gels. The succession of phylotypes in the cultures is summarized for the main phylotypes (Table 1). Phylotype F128 (GenBank accession no. DQ289519) retrieved from the in situ sample could not be detected in the cultures after isolation. Phylotype G003 occurred in all cultivation steps. Some phylotypes could be retrieved after isolation but not during further cultivation (G046, G048, G049, G061, G062). Phylotype G037 was detected in all cultures after isolation. Some phylotypes were detected exclusively in steps 3 and 11 (G006, G009, G010, G020), whereas phylotype G372 was detected in some cultures solely in step 11. Some phylotypes are not displayed in Table 1, as they occurred in few samples (G001, G012, G021, G024, G356) (Fig. 1).

Since microalgae were isolated by the technique of micropipetting, it is likely that identified phylotypes originated from the in situ community. With reference to our study, the coherence of in situ detection and recovery during cultivation could be shown for phylotypes G020 and G046.

If we presume that low abundance led to nondetection of phylotypes, those associated bacteria which were not detected in situ but were detected later must have grown rapidly during cultivation, probably due to high substrate concentrations. This has been suggested for some *Gammaproteobacteria* spp. of Helgoland Roads (5). In our study, this might be the case for several phylotypes. Relatives of these have been found in microalgal cultures retrieved from Helgoland Roads (Fig. 2) (17). It is assumed that those bacterial groups are important for the interaction of bacteria and phytoplankton. Effects of *Alteromonas* sp. beneficial to microalgae have already been shown previously (20), supporting our assumption.

It has not yet been possible to successfully identify bacterial species attached directly on algal cells; therefore, the possibility cannot be excluded that the detected phylotypes belong to opportunists. These might profit from cultivation conditions. It is possible that the shifts might also result from confinement (6, 13, 18). Furthermore, the dilution after each recultivation step might have favored shifts in community composition.

In conclusion, the process of cultivation did not lead to species-specific associated bacterial communities. Findings from culture experiments should be scrutinized regarding cultivation impacts, and future analysis of bacterium-phytoplankton associations should comprise experiments and field studies. In particular, a combination of fingerprinting methods and multivariate statistics provides a promising tool to achieve further insights into phytoplankton-bacterioplankton interactions (15, 16).

TABLE 1. Appearance of phylotypes within cultivation steps

| Phylotype | Identification | | |
|---|---|---|---|
| | Cultivation step(s) | Phylogenetic group | Microalga |
| F128 | In situ | *Bacteroidetes* | |
| G046 | In situ | *Alphaproteobacteria* | |
| | 1 | *Alphaproteobacteria* | *T. rotula* |
| | 1 | *Alphaproteobacteria* | *P. pungens* |
| | 1 | *Alphaproteobacteria* | *G. delicatula* |
| | 1 | *Alphaproteobacteria* | *S. costatum* |
| G020 | In situ | *Bacteroidetes* | |
| | 3 | *Bacteroidetes* | *T. rotula* |
| | 11 | *Bacteroidetes* | *P. pungens* |
| | 3 | *Bacteroidetes* | *G. delicatula* |
| G048 | 1 | *Gammaproteobacteria* | *T. rotula* |
| | 1 | *Gammaproteobacteria* | *P. pungens* |
| | 1 | *Gammaproteobacteria* | *G. delicatula* |
| | 1 | *Gammaproteobacteria* | *S. costatum* |
| G049 | 1 | *Gammaproteobacteria* | *T. rotula* |
| | 1 | *Gammaproteobacteria* | *P. pungens* |
| | 1 | *Gammaproteobacteria* | *G. delicatula* |
| | 1 | *Gammaproteobacteria* | *S. costatum* |
| G061/G062 | 1 | *Gammaproteobacteria* | *T. rotula* |
| | 1 | *Gammaproteobacteria* | *P. pungens* |
| | 1 | *Gammaproteobacteria* | *G. delicatula* |
| | 1 | *Gammaproteobacteria* | *S. costatum* |
| G037 | 1 | *Gammaproteobacteria* | *T. rotula* |
| | 1 | *Gammaproteobacteria* | *P. pungens* |
| | 1, 3 | *Gammaproteobacteria* | *G. delicatula* |
| | 1 | *Gammaproteobacteria* | *S. costatum* |
| G003 | 1, 3, 11 | *Alphaproteobacteria* | *T. rotula* |
| | 1, 3, 11 | *Alphaproteobacteria* | *P. pungens* |
| | 11 | *Alphaproteobacteria* | *G. delicatula* |
| | 11 | *Alphaproteobacteria* | *S. costatum* |
| G006 | 11 | *Alphaproteobacteria* | *T. rotula* |
| | 3, 11 | *Alphaproteobacteria* | *P. pungens* |
| | 11 | *Alphaproteobacteria* | *S. costatum* |
| G009 | 3, 11 | *Alphaproteobacteria* | *T. rotula* |
| | 11 | *Alphaproteobacteria* | *P. pungens* |
| | 3, 11 | *Alphaproteobacteria* | *G. delicatula* |
| | 3, 11 | *Alphaproteobacteria* | *S. costatum* |
| G010 | 3, 11 | *Gammaproteobacteria* | *T. rotula* |
| | 11 | *Gammaproteobacteria* | *P. pungens* |
| | 3, 11 | *Gammaproteobacteria* | *G. delicatula* |
| | 3, 11 | *Gammaproteobacteria* | *S. costatum* |
| G372 | 11 | *Alphaproteobacteria* | *T. rotula* |
| | 11 | *Alphaproteobacteria* | *P. pungens* |
| | 11 | *Alphaproteobacteria* | *G. delicatula* |
| | 11 | *Alphaproteobacteria* | *S. costatum* |

**Nucleotide sequence accession numbers.** Sequences obtained in this study are available from GenBank (DQ376145 to DQ376174).

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## REFERENCES

1. **Bell, W., and R. Mitchell.** 1972. Chemotactic and growth responses of marine bacteria to algal extracellular products. Biol. Bull. **143:**265–277.
2. **Bidle, K. D., and F. Azam.** 2001. Bacterial control of silicon regeneration from diatom detritus: significance of bacterial ectohydrolases and species identity. Limnol. Oceanogr. **46:**1606–1623.
3. **Clarke, K. R., and R. M. Warwick.** 2001. Change in marine communities: an approach to statistical analysis and interpretation. PRIMER-E, Plymouth, United Kingdom.
4. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31:**442–443.
5. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044–3051.
6. **Ferguson, R. L., E. N. Buckley, and A. V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47:**49–55.
7. **Green, D. H., L. E. Llewellyn, A. P. Negri, S. I. Blackburn, and C. J. S. Bolch.** 2004. Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*. FEMS Microbiol. Ecol. **47:**345–357.
8. **Grossart, H. P., F. Levold, M. Allgaier, M. Simon, and T. Brinkhoff.** 2005. Marine diatom species harbour distinct bacterial communities. Environ. Microbiol. **7:**860–873.
9. **Guillard, R. R. L.** 1975. Culture of phytoplankton for feeding marine invertebrates. *In* W. L. Smith (ed.), Culture of marine invertebrate animals. Plenum Press, New York, NY.
10. **Hold, G. L., E. A. Smith, M. S. Rappé, E. W. Maas, E. R. B. Moore, C. Stroempl, J. R. Stephen, J. I. Prosser, T. H. Birkbeck, and S. Gallacher.** 2001. Characterisation of bacterial communities associated with toxic and non-toxic dinoflagellates: *Alexandrium* spp. and *Scrippsiella trochoidea*. FEMS Microbiol. Ecol. **37:**161–173.
11. **Jasti, S., M. E. Sieracki, N. J. Poulton, M. W. Giewat, and J. N. Rooney-Varga.** 2005. Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium* spp. and other phytoplankton. Appl. Environ. Microbiol. **71:**3483–3494.
12. **Kaczmarska, I., J. M. Ehrman, S. S. Bates, D. H. Green, C. Léger, and J. Harris.** 2005. Diversity and distribution of epibiotic bacteria on *Pseudonitzschia multiseries* (Bacillariophyceae) in culture, and comparison with those on diatoms in native seawater. Harmful Algae **4:**725–741.
13. **Lee, S. H., and J. A. Fuhrman.** 1991. Species composition shift of confined bacterioplankton studies at the level of community DNA. Mar. Ecol. Prog. Ser. **79:**195–201.
14. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüβmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. A software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
15. **Rooney-Varga, J. N., M. W. Giewat, M. C. Savin, S. Sood, M. LeGresley, and J. L. Martin.** 2005. Links between phytoplankton and bacterial community dynamics in a coastal marine environment. Microb. Ecol. **49:**163–175.
16. **Sapp, M., A. Wichels, K. H. Wiltshire, and G. Gerdts.** 2007. Bacterial community dynamics during winter-spring transition in the North Sea. FEMS Microbiol. Ecol. **59:**622–637.
17. **Sapp, M., A. S. Schwaderer, K. H. Wiltshire, H. G. Hoppe, G. Gerdts, and A. Wichels.** 31 January 2007, posting date. Species-specific bacterial communities in the phycosphere of microalgae? Microb. Ecol. doi:10.1007/s00248-006-9162-5.
18. **Schäfer, H., P. Servais, and G. Muyzer.** 2000. Successional changes in the genetic diversity of a marine bacterial assemblage during confinement. Arch. Microbiol. **173:**138–145.
19. **Schäfer, H., B. Abbas, H. Witte, and G. Muyzer.** 2002. Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. FEMS Microbiol. Ecol. **42:**25–35.
20. **Stewart, J. E., L. J. Marks, C. R. Wood, S. M. Risser, and S. Gray.** 1997. Symbiotic relations between bacteria and the domoic acid producing diatom *Pseudonitzschia multiseries* and the capacity of these bacteria for gluconic acid/gluconolactone formation. Aquat. Microb. Ecol. **12:**211–221.
21. **Wichels, A., C. Hummert, M. Elbrächter, B. Luckas, C. Schütt, and G. Gerdts.** 2004. Bacterial diversity in toxic *Alexandrium tamarense* blooms off the Orkney Isles and the Firth of Forth. Helgol. Mar. Res. **58:**93–103.
22. **Wiltshire, K. H., and C. D. Durselen.** 2004. Revision and quality analyses of the Helgoland Reede long-term phytoplankton data archive. Helgol. Mar. Res. **58:**252–268.