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# *Technical Note:*

# Purification of benthic diatoms from associated bacteria using the antibiotic imipenem

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**Benthic diatoms and bacteria often co-operatively build up phototrophic epilithic biofilms. Studying the properties and contributions of the individual partners requires the establishment and maintenance of axenic cultures of the involved organisms. Axenification of biofilm organisms is often challenging, because bacteria as well as diatom cells are embedded in a matrix of extracellular polymeric substances (EPS). Due to this mucilage, the cells stick together and also are less affected by antimicrobial substances. Here we describe a short and feasible protocol for culture axenification, which was successfully applied to cultures of the benthic diatoms *Achnanthidium minutissimum*, *Cymbella affiniformis* and *Nitzschia palea*. Our protocol includes treatment of the cultures with the antibiotic imipenem and might also be useful for the purification of other cultivated diatom strains. Once axenified, diatom cultures often decay after a certain life span. Our protocol is especially useful to re-establish axenic cultures from co-cultures of diatoms with their accompanying bacteria (also referred to as xenic cultures).**

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## Introduction

Epilithic biofilms consist of a complex community of algae and bacteria, which thrive within a shared matrix of extracellular polymeric substances (EPS) that, together with the cells, makes up the biofilm (Callow and Callow 2006; Flemming and Wingender 2010). Among the eukaryotic algae found in such biofilms, diatoms are particularly abundant (Callow and Callow 2006). To understand the organismic interactions that lead to the formation of biofilms, the generation and maintenance of axenic diatom cultures from environmental samples is a basic prerequisite. However, biofilm-forming benthic diatoms often are difficult to separate from their bacterial partners. Purification via simple application of antibiotics often turned out to be ineffective, because microorganisms in biofilms often are less sensitive to antimicrobial agents compared to their planktonic counterparts (Ceri et al. 1999). A reduced penetration of substances into the cells due to the diffusion barrier of the biofilm matrix could be one possible explanation for this phenomenon (Stewart 1996; Stewart and William Costerton 2001). The EPS formation of diatoms is influenced by the associated bacterial community (Bruckner et al. 2008). This implies that the effectiveness of a purification method depends not only on the diatom species itself, but also on the composition of the associated bacterial community.

A variety of methods for establishing axenic diatom cultures have been reported, applying a diverse range of techniques, like mechanical separation of cells from the surrounding biofilm matrix, filtration, treatment with detergents, substitution of the associated bacteria with cultivated bacteria that are sensitive against antibiotics and subsequent removal of these bacteria (Bruckner and Kroth 2009; Shishlyannikov et al. 2011). All these axenification techniques either start from environmental samples, or from cultures of diatoms that still contain the associated bacteria that have been co-isolated along with the diatoms (so called xenic cultures) (Bruckner and Kroth 2009). Depending on the species, after a certain time of axenic cultivation, some diatom cultures lose their viability. Xenic cultures are not only easier to maintain than axenic cultures, but also more stable during long term cultivation. It is therefore a good strategy to maintain a xenic culture and to derive axenic sub-cultures as needed for experimental reasons. In this study we present a protocol for the purification of axenic cultures out of xenic cultures which was successfully applied to three different benthic diatom species.

### Initial cultivation of diatom strains

*Achnanthidium minutissimum* (Kützing) Czarnecki isolate B-13, *Cymbella affiniformis* Krammer isolate B-16 and *Nitzschia palea* (Kützing) W. Smith isolate B-01 were isolated in January 2009 from phototrophic, epilithic biofilms taken from the littoral zone of Lake Constance (47°41´N; 9°11`E, Germany) with the methods described in Bruckner and Kroth (2009). Biofilms were scraped from stone surfaces and about 200 µl of the material was diluted in 1-1.5 ml Bacillariophycean Medium (BM). This medium (Schlösser 1994) was modified by the following changes: 236 µM $Na_2CO_3$, 27 µM Iron(III) citrate hydrate, 214 µM citric acid, 60 nM $MnSO_4$, 200 µg/l Thiamine-HCl and 10 µg/l Biotin were used. The biofilm suspension was vortexed for 10 min, diluted and 100 µl were streaked on BM agar plates containing 1.5% bacto-agar (Becton, Dickinson and Company, USA). After cultivation at standard conditions for diatoms (16°C, 12:12 light: dark cycle with light intensities of 20 - 50 µmol $m^{-2}s^{-1}$), the emerging diatom colonies were picked and transferred into liquid medium. The diatom strains were taxonomically identified based on morphological aspects and via 18S rDNA sequence analysis.

The diatom isolates were maintained with their naturally occurring bacterial community in xenic cultures.

### Strain axenification

Starting from the xenic cultures, we purified the diatoms roughly following the strategies described by Bruckner and Kroth (2009). Our protocol combines mechanical cell separation, removal of loosely attached EPS and treatment with antibiotics as outlined in the protocol box.

In order to overcome the protective effect of the extracellular carbohydrate matrix we loosened the biofilm structure via sonication (Kobayashi et al. 2009; Brown and Bischoff 1962) and EPS dissolution (Staats et al. 1999) as first purification steps. 1-3 ml of the xenic diatom cultures were harvested by centrifugation for 5 min at 3000 g for *A. minutissimum* and *N. palea* and 5000 g for *C. affiniformis*, respectively, and resuspended in 1 ml BM. Cell suspensions were treated with ultrasound (UP50H - Compact Homogenizer, Hielscher Ultrasound Technology, Teltow, Germany) for 30 s in the case of *A. minutissimum*, 60 s for *C. affiniformis* and 10-30 s for *N. palea*, with the instrument's settings at 40% amplitude and 0.5 s frequency. 500 µl of the cell suspension were washed twice with BM, diluted 1:10 and incubated with 98 µg/ml of the ß-lactam antibiotic imipenem (Sigma-Aldrich, Germany) for 2-15 days at the same cultivation conditions as used for the xenic cultures.

In parallel approaches, loosely attached EPS were removed via incubation at 30°C for 2 h with shaking at 500 rpm directly after ultrasonic treatment. We suppose that the necessity of this step may depend on the biofilm structure and the respective species. According to our experience, the removal of EPS using the described methods is not harmful to a number of diatom species (Bruckner and Kroth 2009; this study). Cells were then washed three times, diluted 1:10 and incubated with 75 µg/ml of imipenem for 2-15 days. All approaches were pre-screened continuously at 400x magnification using an inverted optical microscope (Axiovert 40 C, Carl Zeiss MicroImaging GmbH, Göttingen, Germany); cultures without bacteria were further cultivated. This alternative step was especially helpful when purifying *A. minutissimum* and *N. palea*. We chose the antibiotic imipenem, because ß-lactam antibiotics inhibit the cell wall synthesis of bacteria and we therefore expected less toxic effects on chloroplasts as reported for other antibiotics (Shishlyannikov et al. 2011). Imipenem has a broad antimicrobial spectrum (Kropp et al. 1985) and was already successfully used to generate axenic cyanobacterial cultures from environmental isolates (Ferris and Hirsch 1991; Hong et al. 2010). In the first days after antibiotic treatment, the viability of the diatom cells was observed daily via inverse light microscopy, later on in irregular time intervals. *Nitzschia palea* and *A. minutissimum* did not show any microscopically observable morphological changes after treatment with imipenem and could be stably cultivated afterwards, suggesting that the diatom cells remained viable even after a long time of incubation (15 days) with this antibiotic. *C. affiniformis* isolate B-16, however, seems to be more sensitive against imipenem; here we observed many dead cells and empty frustules after an incubation period of 2 days. However, this culture was then able to regenerate in antibiotic-free medium and residual contaminations could be removed by an addition of 150 µg/ml of the ß-lactam antibiotic carbenicillin (Duchefa Biochemie, Netherlands). After imipenem treatments we therefore recommend a post-treatment with other ß-lactam antibiotics like for example carbenicillin, ampicillin, ticarcillin and cefotaxim, or a mixture of these drugs in case that bacterial contaminations should persist after treatment with imipenem. A repetition of the procedure with *A. minutissimum* from two separately cultivated xenic cultures confirmed our data although in one of these cultures bacterial cells remained visible when screened with inverse light microscopy. We speculate that in this culture the bacterial composition may have changed during separate cultivation for several months. Although the effectiveness of a purification method mainly depends on the individual diatom species itself, the composition of the associated bacterial community is apparently also relevant. For example, the EPS formation of diatoms is influenced by the bacterial composition (Bruckner et al. 2008) and thus may change the effective-ness of the following treatment with antibiotics.

The axenic cultures were further cultivated in antibiotic free medium at the same standard cultivation conditions as the xenic cultures.

### Microscopic surveillance of axenity

All approaches were microscopically pre-screened as described above and cultures without visible bacteria were selected for further checks. The respective cells were stained with SYBR Green I (Cambrex, Rockland, ME USA) and screened for bacterial contaminants at 800x magnification with an Olympus BX51 epifluorescence microscope (Olympus Europe, Hamburg, Germany) equipped with the filter set 41020 (Chroma Technology Corp, Rockingham, VT, USA). We could demonstrate that bacterial cells as well as algal nuclei are labelled in the xenic cultures, while in the axenic cultures only the fluorescence of the nuclear DNA of the diatom cells is visible (Figure 1).

### Surveillance of axenity via growth tests

The axenic state of the purified *A. minutissimum* culture was additionally verified by two different methods: The culture was transferred to different bacterial media like solid media with 1.5% Agar-Agar Kobe I (Roth, Germany) containing either diluted LB medium (25 or 50% (v/v)) or medium B (Jagmann et al. 2010) supplemented with 0.05% Trypton, 0.0005% yeast extract and 10 mM HEPES pH 6.8. After three days of cultivation at 16°C the plates were screened for bacterial growth. Additionally, 100 µl of the cultures were cultivated in liquid 50% (v/v) LB for five days at 20°C on a shaker (135 rpm). The xenic cultures quickly formed a bacterial lawn when spread on agar plates with bacterial media and dense bacterial growth could also be observed in the liquid medium. The axenic cultures did not show any bacterial growth on solid or liquid media.

### Molecular biological surveillance of axenity

In axenic cultures, the only 16S rDNAs present should originate from the plastids and the mitochondria of the diatom species, whereas in xenic cultures also other 16S rDNAs may be present. We therefore extracted genomic DNA from xenic and axenic strains using a modified protocol from Murray and Thompson (1980). Cell pellets of 7-8 ml culture aliquots were pestled in 2x CTAB-extraction buffer with 1% 2-mercaptoethanol and processed as described in Bruckner et al. (2008). Precipitation was increased by adding one additional volume of isopropanol. Subsequently, we generated 16S rDNA clone libraries as described in Bruckner et al. (2008). From nine randomly selected colonies, the plasmids containing the 16S rDNA fragments were prepared (QIAprep Spin Miniprep Kit, QIAGEN GmbH, Hilden, Germany), amplified and analysed by Restriction Fragment

Length Polymorphism (RFLP) using the restriction enzymes MspI and AluI (Fermentas, Germany). rDNA fragments were separated by electrophoresis with 4% Sieve Agarose 3:1 (Biozyme Scientific GmbH, Germany). 16S rDNA fragments of the xenic culture, which showed different restriction patterns in RFLP analysis, were sequenced using the T7 promoter primer (GATC, Germany). We also sequenced five fragments cloned from the axenic *A. minutissimum* culture in the same way. BLAST searches were performed against the NCBI database (http://www.ncbi.nlm.nih.gov/). In the xenic cultures, we identified 16S rDNA fragments of *Bacteroidetes* and of β-*Proteobacteria*, while in the axenic cultures only 16S rDNA fragments of diatom plastid DNA were detected. This way we were able to demonstrate the absence of bacterial contaminations in the purified culture.

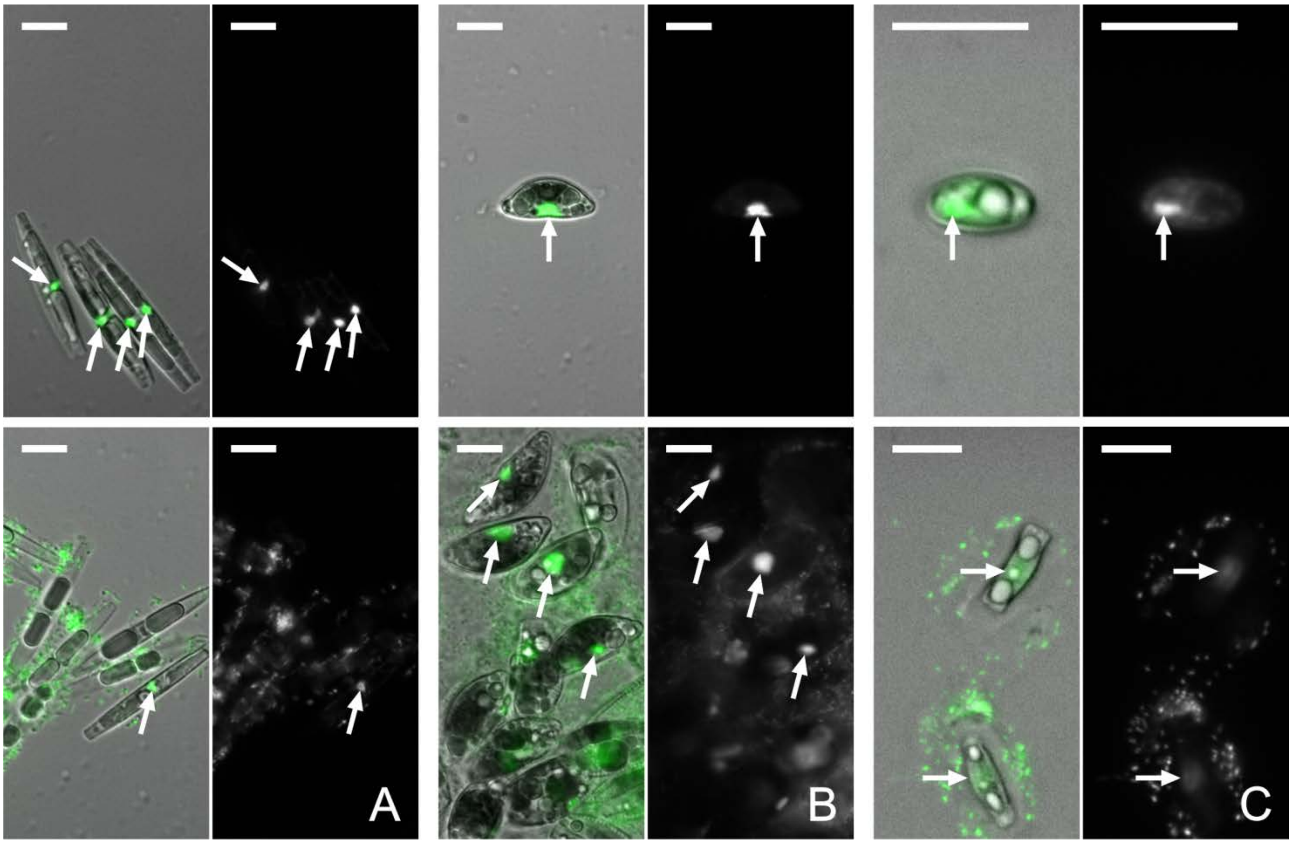

**Figure 1: DNA staining of the xenic and axenic diatom cultures.** (A) *N. palea*, (B) *C. affiniformis*, (C) *A. minutissimum*. Upper row shows the axenic diatom cultures, lower row the corresponding xenic cultures. Left images show the merged transmitted light (grey-scale) and SYBR Green fluorescence (green), right images show SYBR Green fluorescence (grey-scale). Arrows mark the SYBR Green fluorescence of nuclei of diatoms. Scale bars denote 10 µm.

## Conclusions

With this report we demonstrate the usability of the ß-lactam antibiotic imipenem as a new tool for the repeated purification of the diatoms *Achnanthidium minutissimum*, *Cymbella affiniformis* and *Nitzschia palea* from xenic cultures. Generally, treatment with ß-lactam antibiotics such as imipenem in combination with preceding EPS removal might also prove effective for the purification of other diatom species. Our protocol is particularly useful to re-establish axenic cultures from the long term stable xenic cultures of the strains whenever axenic cultures are needed.

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## BOX – axenification protocol

All steps were performed under sterile conditions using a laminar flow and sterile consumables. For the detailed individual experimental procedures see text.

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|---|---|
| **Step 1** | Transfer the diatom cells into new medium |
| **Step 2** | *Sonication* – Treat cell suspension for 10, 30 or 60 sec with ultrasound |
| **(Step 3)** | *Dissolution of EPS* – Alternative step<br>Incubate cell suspension at 30°C and 500 rpm for 2 h |
| **Step 4** | *Washing step* – Wash 2-3 times with BM |
| **Step 5** | *Antibiotic treatment* – Dilute the culture 1:10 and incubate the diluted suspension with 75-98 µg/ml imipenem for 2-15 days, add optional aftertreatment with 150 µg/ml carbenicillin<br>Check the vitality of the diatom cells daily by microscopy |
| **Step 6** | Cultivate cells in antibiotic free medium at appropriate cultivation conditions |