**Effect of a transient perturbation on marine bacterial communities with contrasting history**

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Effect of a transient perturbation on marine bacterial communities with contrasting history

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Running head: Transient perturbation of marine bacteria

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Running head: response of marine bacteria to a toluene perturbation

KEY WORDS

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Single strand conformation polymorphism
Resilience
ABSTRACT

Aims: To compare the resilience of bacterial production of coastal and offshore marine bacterial communities when exposed to a toluene perturbation.

Methods and Results: Chemostats were inoculated with coastal and offshore communities. Bacterial density and production increased before stabilising, and this response to confinement was more marked in the offshore chemostats. Before the toluene perturbation the community structure in the coastal chemostats remained complex whereas the offshore chemostats became dominated by *Alteromonas* sp. After the perturbation, bacterial production was inhibited before peaking briefly at a level 5 fold to that observed before the perturbation, and then stabilising at a level comparable to that before the perturbation. *Alteromonas* dominated both the coastal and the offshore communities immediately after the perturbation and the coastal communities did not recover their initial complexity after twenty days.

Conclusions: Cell lysis induced by the toluene perturbation favoured the growth of *Alteromonas* which could initiate growth rapidly in response to the nutrient pulse. Mathematical modelling suggests that offshore *Alteromonas* were not able to respond as fast as coastal *Alteromonas* considering their high abundance in the offshore communities before the perturbation.

Significance and Impact of Study: Life strategy of coastal *Alteromonas* may be more adapted to rapidly changing nutrient availability.
INTRODUCTION

The oceans play a major role in the global carbon cycle by the important exchange of carbon dioxide between the ocean and the atmosphere. In the oceans, marine heterotrophic bacteria participate in the carbon cycle by the production of biomass from dissolved organic carbon (DOC) and by the release of carbon dioxide by respiration (Azam 1998). At least 50% of the organic carbon from primary production is remineralized by heterotrophic bacteria (Anderson and Ducklow 2001). Given the key role of heterotrophic bacteria in the functioning of the carbon cycle, it is important to study the mechanisms of their functional resilience (i.e. time required to recover their role of mineralization again after a perturbation).

The resilience of bacterial communities in response to perturbations is well studied in terrestrial environments (see Botton et al. (2006) for a review). Functional resilience has been measured at different levels depending on the definition of the function. Following benzene perturbation of two different soils, the broad-scale function of wheat shoot mineralization was unaffected in both soils, whereas the narrow-niche function of 2,4-dichlorophenol degradation was only recovered in the more diverse soil (Girvan et al. 2005). These results are in agreement with previous studies that showed a positive correlation between diversity and resilience (Griffiths et al. 2000). The functional resilience of a community may also depend on the nature and the duration of the perturbation. Long-term exposure to mercury contamination decreased the diversity and the respiration of soil communities and the community was less resistant to subsequent disturbances when compared to the soil community subjected to a transient perturbation (Müller et al. 2002).

One of the few studies to investigate perturbation of marine aquatic communities demonstrated that the coastal heterotrophic bacteria showed resilience in terms of abundance and biomass but not bacterial production when subjected to long-term polychlorinated biphenols (PCB’s)
exposure (Caroppo et al. 2006). To the best of our knowledge, no study has been carried out to determine the resilience of marine bacterial communities to a transient perturbation.

The effect of perturbations on aquatic microorganisms is frequently studied in experimental enclosures since it is easier to manipulate the experimental conditions. Microcosms such as bottle incubations have been used to explore the changes in the microbial community structure and function in response to nutrient additions (Ovreas et al. 2003) or limitation (Vadstein et al. 2003), changes in mortality due to grazing (Flaten et al. 2003) or viral lysis (Proctor and Fuhrman 1992; Winter et al. 2004; Hewson and Fuhrman 2006). Larger volume enclosures, mesocosms, are also popular for such studies (Schäfer et al. 2000; Schäfer et al. 2001) and are advantageous over bottle incubations because the organisms contained within the larger volume are more likely to be representative of the natural community and they can be sampled over longer periods. An alternative to these closed systems is continuous culture in chemostats which allows microbial populations to be grown under controlled conditions for extended periods (see Hoskisson and Hobbs, (2005) for a review). This has been particularly useful for studying bacteria-grazer relationships in mixed microbial assemblages (Pernthaler et al. 2001; Massana and Jürgens 2003). Efficient grazing of the bacterial biomass was observed but there were no significant changes in the bacterial community composition as indicated by fingerprinting techniques (Massana and Jürgens 2003).

In practice, bacterial cells experience very different conditions in the coastal and offshore environment, causing differences in terms of community structure (Schauer et al. 2000; Ghiglione et al. 2005) and potentially life strategies (Weinbauer et al. 2006). Additionally coastal and offshore communities containing bacteria, diatoms, heterotrophic nanoflagellates and ciliates responded differently to a nutrient pulse (Kress et al. 2005). However, little is known about how
differences in bacterial community composition affect the rate of the bacterial mineralization of organic substrate or their reaction to a transient perturbation.

Here we chose to use marine microbial communities as a model to study resilience because of the potential differences between coastal and offshore bacterial communities. The aim of this study was to determine if the same key species were responsible for the functional resilience of the coastal and offshore communities and to compare the growth properties of the key species to determine if their physiological responses were shaped from the ecosystem history. To achieve this, replicate chemostats were inoculated with coastal and offshore marine bacterial communities. The changes in bacterial community structure after a transient toluene perturbation were monitored by capillary electrophoresis single strand conformation polymorphism (CE-SSCP), and the functional resilience of carbon assimilation into biomass was followed by leucine incorporation and flow cytometry. The experimental data were interpreted with the help of a simple model.
MATERIALS AND METHODS

Experimental design

Seawater from the North West Mediterranean Sea was collected in April 2006 at the coastal station SOLA (5 m depth; (42° 31’ N, 03° 11’ E) located 1.6 km from the shore and from the offshore station MOLA (42°27’ N, 03°31’ E, 80-m depth) located 29 km from the shore. Environmental variables were provided by the French coastal monitoring programme SOMLIT ([http://www.domino.u-bordeaux.fr/somlit_national](http://www.domino.u-bordeaux.fr/somlit_national)) for station SOLA and by the Observation Service of the Oceanological Observatory of Banyuls sur Mer (France) for station MOLA. At SOLA, inorganic nutrient concentrations were 0.53 µmol L\(^{-1}\) for NH\(_4\), 1.82 µmol L\(^{-1}\) for NO\(_3\) and 0.26 for NO\(_2\) and at MOLA, they were 0.26 µmol L\(^{-1}\) for NH\(_4\), 9.23 µmol L\(^{-1}\) for NO\(_3\) and 0.13 µmol L\(^{-1}\) for NO\(_2\). The water temperature was 17°C at SOLA and 15°C at MOLA. Chlorophyll concentrations were 0.87 µg L\(^{-1}\) for SOLA and 0.55 µg L\(^{-1}\) for MOLA. For inoculation into the chemostats, seawater from each station was serially filtered through 3µm pore-size GF/D and 0.8µm pore-size GF/F filters (Whatman, Dassel, Germany) to eliminate grazers and phytoplankton. The seawater medium was prepared by autoclaving seawater from SOLA for 2h at 110 °C and then storing it at 16 °C.

Four all-glass chemostats (seven liters) were inoculated with six liters of filtered seawater. The two chemostats inoculated with the bacterial communities sampled at SOLA are referred to “coastal communities” and the two chemostats inoculated with the MOLA bacterial communities are referred to “offshore communities”. Nutrients were provided continuously from the same bottle of seawater medium with a peristaltic Ecoline pump (Bioblock, Montpellier, France) at the rate of 10 ml per hour (i.e. hydraulic retention time of 25 days, dilution rate of 0.04 d\(^{-1}\)). A filtered (0.2µm pore-size GV filters, Durapore, Millipore Corp.) air system saturated the
chemostats with sterile oxygen. The entire apparatus was maintained in the dark in a constant temperature chamber at 16°C.

**Application and monitoring of the toluene perturbation**

In order to characterize the reaction of the bacterial communities to a perturbation, a temporary pulse of toluene (ACS-iso for analysis, Carlo Erba) at saturating concentrations (10 ml per chemostat) was applied for 24 h on day 13. To monitor toluene concentration, five ml samples were collected twice daily between day 13 and day 17. Most of the toluene was eliminated from the chemostat after this period by stopping the stirring and removing the upper toluene phase by pumping. The remaining toluene was evacuated by the air system within 12 hours due to its high volatility. The toluene concentration was monitored by spectrofluorometry (Shimadzu F-2500, Shimadzu Corp.) as the toluene emits naturally fluorescence when excited at 261 nm. Five ml-samples were collected through a side septum. The same day, two ml aliquots of the sample were excited at 261 nm and fluorescence was collected at 285 nm. The sensitivity of the assay was modified by selection of 400 or 700 volts for the photomultiplicator depending on the intensity of the collected signal, which allowed quantification from five to 2000 µM. For each scale, a calibration curve was performed on a dilution series of toluene.

**Measure of the bacterial production by leucine incorporation**

Before and after the perturbation, bacterial production was measured every one or two days. On the day of the perturbation, bacterial production was measured four hours before and 10 hours after the toluene addition and then twice a day. Bacterial production was estimated from the rates of protein synthesis using \([^3]H\)-L-leucine (Amersham, S.A., 117 Ci mmol\(^{-1}\)) as described before (Simon and Azam 1989; Lebaron et al. 2001) with slight modifications. Duplicate samples (5 ml) and one trichloroacetic acid (TCA)-killed control (5 ml) were incubated with \([^3]H\)-L-leucine
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(4 nmol L\(^{-1}\) final concentration) and 16 nmol L\(^{-1}\) of cold leucine. Samples were incubated in the dark in the same chamber as the chemostats for one to three hours depending on the bacterial production expected from previous experiment. Incubations were terminated by adding TCA (5% final concentration). Subsequently, the samples were incubated at 4°C for at least four hours and then centrifuged at 13000 rpm for 15 minutes. The pellets were gently washed once with one ml TCA (5%) and once with ethanol (70% v/v) without disturbing the pellet (centrifuging at 13000 rpm, 5 min) before resuspending the pellets in liquid scintillation cocktail (FilterCount, Packard) for radioassaying with a scintillation counter LS 5000CE (Beckman). The number of disintegrations per minute was converted into picomoles (pmol) of incorporated leucine.

Quantification of cell numbers and cell size by flow cytometry

Samples of 100 µl were collected on a daily basis from each chemostat, fixed with formaldehyde (2% v/v final concentration) and the cells stained with 2.5µl of a 1/10,000 dilution of SYBRGreenI (Molecular Probes) for 20 min at room temperature in the dark. Counts were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon laser (488 nm, 15 mW) as described previously (Vázquez-Dominguez et al. 2005). Stained bacterial cells, excited at 488 nm, were enumerated according to their side scatter (SSC) and green fluorescence (FL1) collected at 450/50 nm. CellQuest software (Becton Dickinson) was then used to provide mean values of bacterial density and scatter. Fluorescent beads (0.94 µm) (Polysciences Inc., Warrington, PA, USA) were added to each sample to normalize cell fluorescence emission and scatter (rSSC) values among samples.

Monitoring of changes in bacterial community structure by SSCP analysis

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For SSCP analysis, 100-200 ml samples were collected from each chemostat every two to four
days by stopping the output pump for a few hours and then setting a high flow rate to pump the
accumulated water. The samples were filtered into 0.2 µm pore-size Sterivex filter units
(Millipore) and stored at -20°C before DNA extraction. Cell lysis was accomplished according to
the protocol of Ghiglione et al. (2005) with slight modifications: An initial incubation for 45 min
at 37°C was performed after adding 50 µl of freshly prepared lysosome solution (20 mg ml\(^{-1}\)) and
1.6 ml of lysis buffer (50 mM Tris hydrochloride pH 8.3, 40 mM EDTA, 0.75 M sucrose). A
second incubation at 50°C was performed for 1 h after adding 100 µl of 10% sodium dodecyl
sulfate and 10 µl of proteinase K (20 mg ml\(^{-1}\)). The lysate was then purified using the DNeasy
tissue Kit (Qiagen) (Elution in 100 µl EB).

For SSCP analysis, a short fragment (205bp) of the V3 region of the 16S rRNA gene was PCR
amplified using the universal bacterial primers W49 (ACGGTCCAGACTCCTACGGG); \textit{E.coli}
positions (329-348) and 6-FAM labelled W104 5’(TTACCGCGGCTGCTGGCAC)3’; \textit{E.coli}
positions (515-533) (Eurogentec, Belgium) as described previously (Chachkhiiani et al 2004).
PCR reactions contained 1 µl template DNA, 0.4 µM primers, 0.2 mM dNTPs, 2.5 U \textit{pfu} Turbo
DNA polymerase and 1X \textit{pfu} buffer (Stratagene). Cycling conditions were 2 min at 94°C
followed by 25 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C, with a final extension step
of 10 min at 72°C. Amplification product sizes were confirmed by agarose gel electrophoresis.
PCR products were diluted 10 to 600-fold, and 1 µl of the dilution was denatured in 19 µL
formamide together with the internal standard ROX 400HD (Applied Biosystems) for 10 minutes
at 94°C and rapidly cooled in an ice bath. The sample was then analysed under non-denaturing
conditions by CE-SSCP with an ABI 3100 genetic analyzer (Applied Biosystems) using the
protocol described by Delbès et al (2000). Fingerprint analysis was done with SAFUM, an
adapted software developed with Matlab™ especially for SSCP analysis (Zemb et al. 2007). For
analysis by SAFUM, the raw data corresponding to the four channels stored in the fsa files generated by the Genscan Analysis Software were exported using Chromagna® (Fekete et al. 2003) into csv files. These files were then imported into SAFUM which normalized the total area of the SSCP profiles and the mobilities between different runs using the internal standard.

Assignation of the major SSCP peak by cloning and sequencing of 16S rRNA genes

Almost full length 16S rRNA genes were PCR amplified from DNA samples extracted from a coastal and an offshore chemostat after 18 days using the universal bacterial primers 27F_mod (5’-AGRGTGTATGCMTCGGCTCAG) and 1492_mod (5’-TACGGYTACCTTGTTAYGACTT) (Vergin et al. 1998). For each DNA sample, 5 replicate PCR reactions were made to reduce PCR bias. Each 50 µl PCR reaction contained 1 µl template DNA together with 0.1 µM primers, 0.8 mM dNTPs, 2U of SuperTaq Polymerase and 1X SuperTaq buffer (HT Biotechnology, Cambridge, UK). The reactions were cycled using the following parameters: an initial denaturation of 3 min at 94 °C followed by 25 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C with a final extension of 10 min at 72 °C. To reduce the presence of heteroduplexes, the replicate PCR reactions were pooled and purified with the Qiaquick PCR purification kit (elution in 50µl EB buffer) before undergoing reconditioning PCR (Thompson et al. 2002). Briefly, 10 µl of purified products were added to 90 µl of fresh PCR mix before cycling a further 5 times with the above PCR parameters. The presence of a single band of the correct size was verified by agarose gel electrophoresis. PCR products were cloned the same day using the TOPO TA cloning kit (pCR2.1 vector; Invitrogen) according to the manufacturer’s instructions. Ten white clones were picked from each of the 2 clone libraries for Restriction Fragment Length Polymorphism (RFLP) screening before sequencing. Inserts from the clones were PCR amplified directly from E.coli cells by using the M13F and M13R primers. Each 25 µl PCR reaction contained 1 µM
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primers, 0.8 mM dNTPs, 1U of SuperTaq Polymerase and 1X SuperTaq buffer. *E.coli* cells were
inoculated into the PCR reactions with a toothpick and the reactions were cycled using an initial
denaturation of 7 min followed by 25 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C
with a final extension of 5 min at 72 °C. PCR products (5µl) were digested with 5 U of the
restriction enzymes *Hha*I or *Rsa*I (Promega) with 1X enzyme buffer and 10 mM BSA at 37 °C
overnight and the fragments separated by electrophoresis in 2% agarose gels. RFLP patterns were
compared manually and the clones assigned to different RFLP groups based on the restriction
patterns of both enzymes. One representative of each RFLP group for each sample was selected
for full length sequencing using the primers E785F (5’GGATTAGATACCCCTGGTAGT) and
907R (5’CCGTCAATTCCTTTGAGTTT). Sequences were corrected manually and assembled
using the sequence editing software Bioedit. The sequences were then submitted to BlastN
(Altschul et al. 1997) to find the closest relative. The sequence data from this study have been
submitted to the GenBank database under the accession numbers EU338363-EU338371.

Model Description

The model used to interpret the data is based on processes and describes carbon flow via
differential equations. This allows a quantitative comparison of biomass production with the
biomass lost through dilution. Each simulation of time dependant population consists of three
distinct steps:

1) Simulation of the biomass and the available substrate using the primary model, 2) conversion
of this simulated biomass into cell density using estimates of cellular carbon content, and 3) conversion of the simulated biomass and substrate into incorporated leucine using a secondary model.
Initially, the primary model was used to simulate dilution, uptake, respiration and release of organic matter from the toluene-lysed cells and assimilation of this matter by bacterial strain affiliated to the *Alteromonas* genus in the chemostat. Therefore, the three state variables in the primary model were: dissolved organic (DOC) carbon, complex bacterial biomass and *Alteromonas* biomass. Complex bacterial biomass was neglected for offshore communities because the community was dominated by a single peak assigned as *Alteromonas*. The state variables are expressed in the same units of mass (µg of carbon L\(^{-1}\)) and all equations describe rates of carbon flow in units of mass per unit time (Table 1). Parameter values used are theoretical estimates based on the ranges of empirically determined values obtained from the literature. (Table 2).

Secondly, the modelled biomass was converted in observable bacterial density using the cellular carbon content and the relative side scatter (rSSC) value observed by cytometry. This conversion was done using the relationships for C (carbon content per cell in fg C cell\(^{-1}\)) and V (biovolume in µm\(^3\)), determined by Troussellier et al. (1999) and Azam et al. (1983): (1) \( V = 8.06 \times \text{rSSC} - 0.02 \) and (2) \( C = 92 \times V^{0.598} \). For rSSC higher than 0.07, carbon content per cell was assumed to be equal to 68 µg.

Thirdly, the measured incorporation of leucine was compared to the expected incorporation given the simulated biomass and substrate using a secondary model. This secondary model was designed so that the lytic effect of the toluene was not neglected during the incubation time with the labelled leucine. The parameters for the bacterial growth are the same as those used for the primary model. The constant needed to convert biomass into leucine consumption was taken from the literature (2005). Equations of this secondary model are summarized in Table 1.

The equations (Table 1) were implemented in Matlab\textsuperscript{TM} and resolved using the ODE45 algorithm.
RESULTS

Stabilization and reaction of coastal communities to the perturbation

For the coastal communities, the bacterial density increased slightly during the first 13 days. The continuous input of substrate sustained an average cellular density in both coastal communities of 1.39 ± 0.4 $10^6$ cells ml$^{-1}$ and a bacterial production of 57 ± 37 pmol leu L$^{-1}$ h$^{-1}$ between the day 4 and the day 13 (Figure 1) despite the daily output of 240 ml. Mean cell size was evaluated at 0.45 µm$^3$ (i.e. cellular carbon content of 40 fg).

Figure 2 shows the development of the bacterial community structure with time before and after the toluene perturbation as revealed by SSCP analysis. After four days, the profiles from the replicate chemostats showed common peaks but the relative abundances of the major peaks were different from each other. However, after nine days the SSCP profiles of the replicate chemostats were very different. Furthermore, for a given chemostat, the SSCP profiles varied considerably over time during the first nine days. After the toluene perturbation, only a single peak was detected and remained dominant until the end of the experiment. After the addition of toluene, no leucine incorporation was detectable and the bacterial density decreased to 2.29 ± 1.18 $10^5$ cells ml$^{-1}$. This reduction in cell density was accompanied by a reduction in the profile diversity from 25 peaks to 1 or 3 depending on the chemostat (Figure 2). Two days after the perturbation, one population of larger cells (cellular carbon content estimated at 60 fg) emerged concomitantly (data not shown) with one dominant phylotype and the bacterial production increased dramatically to 1576 ± 22 pmol leu L$^{-1}$h$^{-1}$ three days after the perturbation. Seven days after the perturbation, the bacterial production stabilized at 430 ± 98 pmol leu L$^{-1}$h$^{-1}$ (Figure 1). The main feature of the coastal communities was a pronounced rebound of the productivity after the perturbation that coincided with a dominant phylotype that was obviously favoured after the perturbation.
Stabilization and reaction of offshore communities to the perturbation

For the offshore communities, the cellular density first increased dramatically during the first four days of incubation from $1.88 \pm 0.4 \times 10^5$ to $2.94 \pm 0.5 \times 10^6$ cells mL$^{-1}$ that was sustained between the day 4 and 13 (Figure 1), which was twice as much as the density sustained in the coastal communities. The bacterial production was $410 \pm 118$ pmol leu L$^{-1}$h$^{-1}$, which was 8 times higher than the production observed for coastal communities (Figure 1). The change in bacterial community structure is shown in figure 2. As the SSCP profiles between the replicate chemostats were identical at each time point, a single replicate is shown. In contrast to the coastal communities that remained complex, the SSCP profiles became dominated by a single peak after 2 days. This peak emerged concomitantly with a single large cell population observed by flow cytometry (data not shown). After the addition of toluene, no leucine incorporation was detectable and the bacterial density decreased by nearly a factor of ten to $3.28 \pm 2.27 \times 10^5$ cells mL$^{-1}$, which is similar to the decrease observed in the coastal communities.

After the elimination of toluene, the functional recovery of the offshore bacteria was faster than for the coastal bacteria. Two days after the perturbation, the bacterial abundance was $7.41 \pm 1.97 \times 10^5$ cells mL$^{-1}$, three times higher than for the coastal communities at the same time. The bacterial production started again one day after the perturbation and for a short period of time attained up to five times the initial production rate three days after the perturbation ($1881 \pm 200$ pmol leu L$^{-1}$h$^{-1}$). The maximal production of offshore communities was higher than for coastal communities, but it was not statistically meaningful due the low number of samples. Seven days after the perturbation, the production stabilized at $485 \pm 128$ pmol leu L$^{-1}$h$^{-1}$ which was identical to the stable production after the perturbation of the coastal communities (Figure 1).
Assignation of the major SSCP peak

The major SSCP peak observed after the toluene perturbation for the coastal chemostats, and before and after the perturbation for the offshore chemostats, was assigned by cloning and sequencing of 16S rRNA genes. The majority of the sequences from both stations (18 out of 20) fell into 4 different RFLP groups. One representative clone from each station within each RFLP group was sequenced, and all these sequences showed 99% identity to an uncultured marine bacterium clone Surf1.42 (accession no. DQ071162) that was related to *Alteromonas* sp. However, the relative abundance of the *Alteromonas* peak at day 0 was undetectable in the coastal community (hidden by neighbour peaks) and only represented about 2% in the offshore chemostats.

Results of the model

To better understand the role of biomass synthesis, two complementary models were created, one that simulated biomass dynamics in the chemostat and one that simulated the labelled biomass dynamics in the incubation performed to measure bacterial production. The presence of two connected models was necessary in order to take the lytic activity of toluene into account during the incubations to measure the bacterial production. We saw that the bacterial production in offshore communities recovered faster than those of coastal communities (Figure 1), but the model suggested that this did not occur as fast as expected in the offshore communities (Figure 3), given their high abundance of *Alteromonadaceae* at day 13 before the perturbation (Figure 20).
DISCUSSION

This study investigated the resilience of bacterial production (ability to recover from a perturbation) of coastal and offshore bacterial communities following a toluene perturbation in replicate chemostats without grazers. The bacterial production was followed by leucine incorporation and the structure of the community was monitored by CE-SSCP. In this study, both communities showed a peak of bacterial production 3 days after the toluene perturbation and this coincided with the dominance of a single SSCP peak assigned as *Alteromonas sp.* A model was designed to understand if the post-perturbation production could be explained quantitatively by assimilation of the organic matter released by the toluene perturbation. This model suggests that the *Alteromonas* strain from offshore water had reacted differently than the strain from coastal waters to a sudden nutrient availability.

**Before the toluene perturbation**

During the first few days of the experiment, there were increases in bacterial cell numbers and bacterial production in both sets of chemostats, though the increase was more pronounced in the offshore chemostats. Such increases in bacterial abundance were already reported for bottle incubation experiments (Eilers et al. 2000) and mesocosm studies (Schäfer et al. 2000; Lebaron et al. 2001; Allers et al. 2007) and are thought to be a response to confinement (Ferguson et al. 1984). SSCP analysis of the bacterial diversity revealed that the important increase in bacterial abundance and production in the offshore chemostats occurred concomitantly with a rapid decrease in the complexity of the profiles which became dominated by one peak after only four days. This was in striking contrast to the coastal chemostats which remained relatively complex up until the perturbation. In addition, these bacterial communities were highly dynamic, exhibiting rapid changes in the relative abundance of particular peaks as well as the disappearance and reappearance of other peaks.
The major peak that rapidly dominated in the offshore chemostats was later assigned as *Alteromonas* sp. Rapid community shifts towards culturable bacteria such as *Alteromonas* in bottle incubations (Eilers et al. 2000), chemostats (Massana and Jürgens 2003) and seawater mesocosms (Pukall et al. 1999; Schäfer et al. 2000; Allers et al. 2007) have been observed frequently. However, the initial high abundances of *Alteromonas* were then followed by a decline in these populations that was correlated with high grazing activity (Schäfer et al. 2001; Allers et al. 2007). Despite the high activity/fast growth rate of *Alteromonas* sp., it is usually found in low concentrations in nature which is thought to be due to tight control by grazers (Beardsley et al. 2003) and probably by viruses. However, high abundances of *Alteromonas* sp. have been recorded in the north-west Mediterranean Sea (Alonso-Saez et al. 2007). In contrast to the offshore chemostats in this study, the dominance of *Alteromonas* in another chemostat experiment was accompanied by relatively high richness of other bacterial species (Massana and Jürgens 2003). This could be explained by the presence of grazers in this latter study, which could have prevented total overgrowth by *Alteromonas*.

In addition to grazing, nutrient concentration is another parameter which influences strongly bacterial community structure (Schäfer et al. 2001; Allers et al. 2007). Using autoclaved seawater medium collected from the coastal station could have stimulated the growth of *Alteromonas* in the offshore chemostats for two reasons. Firstly, autoclaving unfiltered seawater can increase the yield of bacteria in seawater cultures (Ammerman et al. 1984) and secondly, the water from the coastal station probably contained higher concentrations of organic matter and inorganic nutrients than the offshore station and so using this medium for the off-shore chemostats may have also caused a nutrient perturbation. Further experiments should be done to test this hypothesis, by filtering seawater before autoclaving, and by using seawater from the offshore station to feed the offshore chemostats.
After the toluene perturbation

Brief exposure (24h) to saturating concentration of toluene was chosen as perturbation because the toluene could be removed easily by physical means from the chemostat so that biodegradation is not the key factor of resilience and its concentration monitored easily by a fluorescent assay. Furthermore all bacteria were likely to be affected since toluene is soluble in water (0.38 g L\(^{-1}\) at 20°C). Considering the hydraulic retention time, a non-growing population would have decreased by 40% when the shock was applied, which limits the alterations due to removal from potential syntrophy with photosynthetic organisms.

In these experiments, the toluene perturbation totally inhibited bacterial production and growth (Figure 1) and the richness decreased drastically (Figure 2). Interestingly, *E.coli* cells exposed to a similar concentration of toluene lost the ability to synthesise proteins concomitantly with a release of RNA and proteins in the media, even though their membranes were not disrupted (Jackson and DeMoss 1965). We assume that most bacterial cells in the chemostats were permeabilized when exposed to the toluene. The released nutrients could then have been exploited efficiently by *Alteromonas* to outcompete the other phylogenetic groups. Nayar et al. (2005) suggested that phytoplankton exposed to diesel fuel released organic matter that was then assimilated by a few heterotrophic bacteria. The good fit of the model to the coastal chemostat suggest that no complex physiological response is needed to understand the reaction of the coastal communities. In this experiment and after the toluene perturbation, all chemostats were dominated by large, high nucleic acid (HNA) *Alteromonas* sp. bacteria, despite their low initial abundance in the coastal chemostats (Figure 2 M-R). This suggests that some low abundant species can react rapidly to changing conditions (Pernthaler et al. 2001). Indeed, the rapid growth and dominance of *Alteromonas* is congruent with the fact that some *Alteromonas* cells with a
high nucleic acid content survived the perturbation and were able to divide very quickly (Eilers et al. 2000; Pernthaler et al. 2001). Furthermore, the large cell size of *Alteromonas* (Allers et al. 2007) that could have been induced by the released nutrients (phosphate and/or carbon) would then have allowed an increased assimilation of substrate per cell and unit time. This mechanism is supported by research into the life strategies of pelagic microorganisms that showed that the uptake of non-limiting elements led to increases in cell size which in turn allowed a better assimilation of the limiting nutrient via the increased cell surface area (Thingstad et al. 2005).

**Comparison of the communities’ functioning with a simple model**

We built a model based on carbon flow (although carbon was not necessary the unique limiting factor in this experiment) to test if the rapid growth of *Alteromonas* after the toluene perturbation was due to the dominance of this genus before the perturbation. Parameters of the model were taken from literature within a realistic range of values (Table 2). The effect of toluene on cell yield (Volkers et al. 2006) or on carbon input were neglected in our model because toluene was rapidly removed by physical means. As can be seen in Figure 3, our model captures the observations of the coastal communities quite well. However, the model was unable to explain the delayed bacterial production for the offshore communities that were dominated by *Alteromonas* species before the perturbation (Figure 3, bottom). These species from the offshore environment did not recover as fast as theoretically expected from their abundance before the perturbation. The growth of individual cells of the offshore *Alteromonas* was delayed when compared to the cells from the coastal communities. It seems unlikely that this difference was due to different physiological states between coastal and offshore communities since both communities remained in chemostats during 13 days (Berney et al. 2006). Although the *Alteromonas* sequences from the coastal and offshore chemostats showed 99 % similarity, the populations could have possessed different physiologies. It is known that different bacteria with
identical 16S rRNA sequences can possess very different genome sequences and hence different 
physiologies (Jaspers and Overmann 2004). Therefore, we suggest that the frequently perturbed 
coastal area (resuspension and runoff) might be favourable to some microbial strains reacting 
quickier to changing nutrient availability than their offshore relative.

**Comparison with the resilience of other systems**

In our study, the function of the bacterial community could recover but *Alteromonas* dominated 
the community after the perturbation. This is congruent with the case of perturbed denitrifying 
biofilms reported by (Gentile et al. 2006) in which the resilience of the initial structure was never 
observed. This might be due to the short time scale used in our experiments (20 days) and to 
some extent to the absence of predators which play a significant role in shaping bacterial 
communities in natural ecosystems (Steiner et al. 2006). Indeed, the rapid response of 
*Alteromonas* species to changing environmental conditions may be counterbalanced by grazing *in 
situ* (Lebaron et al. 1999; Beardsley et al. 2003; Mohapatra and Fukami 2004). Environmental 
perturbations that affect natural communities generally result in nutrient release whose 
assimilation depends on the configuration of the food web. If the most responsive organisms that 
grow quickly are very sensitive to predation (as it is the case for *Alteromonas* (Schäfer et al. 
2001; Beardsley et al. 2003; Allers et al. 2007)), the dynamics of the predators will obviously be 
a determinant for the resilience of bacterioplankton community structure. Even though we cannot 
rule out the importance of bacterial immigration from the surrounding water *in situ*, it seems 
unlikely because *Alteromonas* reacts within hours. As an example, *Pseudomonas aeruginosa* 
would need approximately 40 hours to reach the middle of our 15-cm wide chemostat (Keller 
1974).

The fact that *Alteromonas* sp was found in all four chemostats after the shock suggests a fairly 
low functional redundancy in the bacterial community.
The fact that the resilience of the bacterial production (i.e., mineralisation) relied exclusively on *Alteromonas* suggests that incorporating *Alteromonas* abundance in models dealing with resilience in marine bacterial communities could be very beneficial. However, the high production at the end of the experiment shows that the composition of the rest of the community was not crucial for the mineralisation resilience.

In conclusion, *Alteromonas* are opportunistic bacteria that can rapidly assimilate released nutrients, thereby causing a rapid resilience of the bacterial production after a perturbation. In this study, the same key species was responsible for functional resilience in coastal and offshore bacterial communities even though the events that these bacteria had previously experienced *in situ* were very different. This suggests that *Alteromonas* could be used as a potential indicator for disturbance events such as recent nutrient release through lytic events, not only in coastal surface water (Allers et al. 2007) but also in offshore waters. The mathematical model used to interpret the data in this study suggested that the offshore *Alteromonas* strains were not able to respond as fast as the strains from coastal waters after the perturbation. Further experiments (such as a pure nutrient perturbation) are required to better understand the differences between coastal and offshore bacteria reported in this study and the role of predation needs to be investigated to evaluate the *in situ* ability of *Alteromonas* as indicator of disturbance.
REFERENCES


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Table 1: Model equations

**Primary model (for the chemostat)**

\[
\frac{dB_a}{dt} = (\mu_a(T) \cdot S - D - \beta_a \cdot T) \cdot B_a
\]

\[
\frac{dB_c}{dt} = (\mu_c(T) \cdot S - D - \beta_c \cdot T) \cdot B_c
\]

\[
\frac{dS}{dt} = D (S_{in} - S) - \alpha_a \cdot \mu_a(T) \cdot S \cdot B_a - \alpha_c \cdot \mu_c(T) S \cdot B_c + \beta_a \cdot T \cdot B_a + \beta_c \cdot T \cdot B_c
\]

**Secondary model (for the leucine incorporation)**

\[
\frac{dB_a^*}{dt} = \mu_a(T) \cdot S \cdot B_a - \beta_a \cdot T \cdot B_a^*
\]

\[
\frac{dB_a}{dt} = - \beta_a \cdot T \cdot B_a
\]

\[
\frac{dB_c^*}{dt} = \mu_c(T) \cdot S \cdot B_c - \beta_c \cdot T \cdot B_c^*
\]

\[
\frac{dB_c}{dt} = - \beta_c \cdot T \cdot B_a
\]

\[
\frac{dS}{dt} = - \alpha_a \cdot \mu_a(T) \cdot S \cdot (B_a + B_c) + \beta_a \cdot T \cdot B
\]

with

- \( B_a \) and \( B_c \) biomass of the total community, of \textit{Alteromonadaceae}, and of complex community excluding \textit{Alteromonadaceae} respectively in \( \mu g \ L^{-1} \)

- \( B_a^*, B_c^* \), the radioactive biomass in \textit{Alteromonadaceae} and in complex biomass in \( \mu g \ L^{-1} \)

- \( \beta_a, \beta_c \), the hypothetical sensitivity to toluene of \textit{Alteromonadaceae} and of complex biomass in \( \mu M^{-1} \ d^{-1} \)

- \( S, S_{in} \), the substrate concentration in the chemostat and in the supply tank in \( \mu g \ L^{-1} \)

- \( D \), the dilution rate in \( d^{-1} \)

- \( T \), the concentration of toluene in \( \mu M \)

- \( 1/\alpha_a, 1/\alpha_c \), the yield coefficient of \textit{Alteromonadaceae} and of the complex biomass

- \( \mu_a(T), \mu_c(T) \), the growth rates of \textit{Alteromonadaceae} and of the complex biomass given the amount \( T \) of toluene
Table 2: Parameters used in the model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Complex consortium sampled at SOLA</th>
<th>Alteromonas sp. sampled at SOLA</th>
<th>Alteromonas sp. sampled at MOLA</th>
<th>Units</th>
<th>Values based on</th>
</tr>
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<tr>
<td>Dilution rate (D)</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>d⁻¹</td>
<td>measure</td>
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<tr>
<td>Substrate input concentration (Sin)</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>µg L⁻¹</td>
<td>Sondergaard and Middelboe (1995) and Sharp (1973)</td>
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<tr>
<td>Initial substrate (So)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>µg L⁻¹</td>
<td></td>
</tr>
<tr>
<td>Initial Biomass (Bo)</td>
<td>37</td>
<td>13</td>
<td>50</td>
<td>µg L⁻¹</td>
<td>Kogure and koike (1987)</td>
</tr>
<tr>
<td>Toluene Threshold (b) that inhibits growth</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>µM</td>
<td>Goodwin et al. (2005)</td>
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<tr>
<td>Growth rate (µ) for [Toluene]&lt;b</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>d⁻¹ µg⁻¹ L⁻¹</td>
<td></td>
</tr>
<tr>
<td>Yield coefficient (1/α)</td>
<td>25%</td>
<td>50%</td>
<td>50%</td>
<td>No unit</td>
<td>Jimenez-Mercado et al. (2007)</td>
</tr>
<tr>
<td>Sensitivity to toluene</td>
<td>7 \times 10⁻⁴</td>
<td>2.8 \times 10⁻⁴</td>
<td>2.8 \times 10⁻⁴</td>
<td>d⁻¹</td>
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<td>Carbon-Leucine conversion factor</td>
<td>1.44 \times 10⁻⁹</td>
<td>1.44 \times 10⁻⁹</td>
<td>1.44 \times 10⁻⁹</td>
<td>g pmol⁻¹</td>
<td>Buesing and Marxsen (2005)</td>
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<td>Cell carbon content</td>
<td>40</td>
<td>60</td>
<td>60</td>
<td>fg</td>
<td>Troussellier et al. (1999) and Azam et al. (1983), Neumann et al. (2005)</td>
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Figure Legends

Figure 1: Experimental bacterial density (A) and production (B) in the chemostats inoculated with coastal bacterial communities (open symbols; triangles for the first chemostat and circles for the second one) or offshore bacterial communities (filled symbols; triangles for the first chemostat and circles for the second one). The toluene addition is represented by an arrow.

Figure 2: SSCP profiles showing the bacterial community structure in the replicate coastal chemostats (A-F and G-L) and one representative offshore chemostat (M-R) during the experiment. The sampling days were 0 (A,G,M), 7 days after inoculation (B,H,N), 9 days after inoculation (C,I,O), 2 days after the perturbation (D,J,P), 9 days after the perturbation (E,K,Q) and 20 days after the perturbation (F,L,R). The areas were normalized by the bacterial density. The peak assigned as *Alteromonas* sp. is indicated with an arrow head. The toluene addition is represented by an arrow.

Figure 3: Modelled biomass, density and production and measured toluene concentration (dashdot line) for coastal communities (A, B and C) and for offshore communities (D, E and F). A, D; correspond to the modelled biomass (solid line) and the modelled substrate (dashed line) in µg of carbon per litre. B, E; correspond to the modelled biomass (solid line) and observed cell density in cells per ml in the first (triangles) and in the second chemostat (circles); C, F; correspond to the modelled production (solid line) and the observed production in pmol of incorporated leucine in first (open symbols) and in the second chemostat (filled symbols).
Figure 1

Bacterial density (10^6 cells mL\(^{-1}\))

Bacterial production (pmol leu L\(^{-1}\) h\(^{-1}\))

Days

Shore

Shore

Offshore

Offshore
Figure 2

<table>
<thead>
<tr>
<th>Day</th>
<th>Shore (1st chemostat)</th>
<th>Shore (2nd chemostat)</th>
<th>Offshore (representative chemostat)</th>
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<tbody>
<tr>
<td>0</td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>H</td>
<td>N</td>
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<tr>
<td>9</td>
<td>C</td>
<td>I</td>
<td>O</td>
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<td>J</td>
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<tr>
<td>20</td>
<td>E</td>
<td>K</td>
<td>Q</td>
</tr>
<tr>
<td>33</td>
<td>F</td>
<td>L</td>
<td>R</td>
</tr>
</tbody>
</table>

Day Shore (1st chemostat) Shore (2nd chemostat) Offshore (representative chemostat)
Figure 3

![Graphs showing bacterial activity over time](image)

- (A) Graph A: Description of bacterial density and biomass over days.
- (B) Graph B: Comparison of experimental data between Shore 1 and Shore 2.
- (C) Graph C: Graph C: Modelled vs. actual bacterial production.
- (D) Graph D: Estimated bacterial biomass over days.
- (E) Graph E: Experimental data for offshore 1 and offshore 2.
- (F) Graph F: Comparison of bacterial production with toluene concentration.

Days