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Biodegradation of the aminopolyphosphonate DTPMP by the cyanobacterium *Anabaena variabilis* proceeds *via* a C-P lyase-independent pathway

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Running head: Polyphosphonate degradation by a cyanobacterium

Originality-Significance Statement

Capability of metabolising the polyphosphonate diethylenetriaminepenta(methylenephosphonic) acid (DTPMP) by the freshwater cyanobacterium *Anabaena variabilis* has been studied in detail. Based on ^{31}P NMR measurements of crude extracts, a pathway for the biodegradation of this xenobiotic bearing five methylenephosphonic groups is proposed. This mechanism differs at least in part from the commonly accepted route for organophosphonate breakdown, which relies on the activity of the inducible C-P lyase complex. Because of the ability to hydrolyze multiple carbon-to-phosphorus bonds in a single molecule, this prokaryotic microalga may be considered a model species for studying the biodegradation of phosphonates.

Summary

Cyanobacteria, the only prokaryotes capable of oxygenic photosynthesis, play a major role in carbon, nitrogen and phosphorus global cycling. Under conditions of increased P availability and nutrient loading, some cyanobacteria are capable of blooming, rapidly multiplying and possibly altering the ecological structure of the ecosystem. Because of their ability of using non-conventional P sources, these microalgae can be used for bioremediation purposes. Under this perspective, the metabolization of the polyphosphonate diethylenetriaminepenta(methylenephosphonic) acid (DTPMP) by the strain CCALA 007 of *Anabaena variabilis* was investigated using ^{31}P NMR analysis.

Results showed a quantitative breakdown of DTPMP by cell-free extracts from cyanobacterial cells grown in the absence of any phosphonate. The identification of intermediates and products allowed us to propose a unique and new biodegradation pathway in which the formation of (*N*-acetylaminomethyl)phosphonic acid represents a key step. This hypothesis was strengthened by the results obtained by incubating cell-free extracts with pathway intermediates. When *Anabaena* cultures were grown in the presence of the phosphonate, or phosphorus-starved before the extraction, significantly higher biodegradation rates were found.

Introduction

Due to their physicochemical properties, polyphosphonates have been found increasing application in many industrial fields, causing their massive release into the environment (Studnik et al., 2015). Because of their effectiveness as ligands for metal ion complexation, these xenobiotics are extensively used in water treatment processes and as additives to many household cleaning products, (May et al., 1986). Among them, diethylenetriaminepenta(methylenephosphonic) acid – DTPMP (Fig. 1), also known as Dequest® 2060S, is widely employed as a general purpose scale inhibitor (especially towards barium sulphate) and a powerful sequestrant in cooling water devices, detergents and bleach, as well as in geothermal and oil field applications (Italmatch-Chemicals, 2014). Organophosphonates are highly water-soluble and stable in aqueous solutions. Moreover, the C-P bond is resistant to chemical hydrolysis, thermal decomposition and photolysis (Ternan et al., 1998), raising concern for

their potential accumulation in aquatic ecosystems (Davenport et al., 2000; Knepper and Weil, 2001; Jaworska et al., 2002). In fact, methods for the effective removal of phosphonates from the environment have yet not been developed. Furthermore, the lack of sensitive analytical methods hampers the assessment of the environmental fate of these compounds (Nowack, 2003).

Fig. 1. Structure of the polyphosphonate diethylenetriaminepenta(methylenephosphonic) acid – DTPMP.

Cyanobacteria, one of the most ancient group of organisms on Earth, have developed an unprecedented ecological plasticity that allows them to colonize virtually any habitat (Whitton and Potts, 2000), from deserts (Bar-Eyal et al., 2015) to glaciers under harmful UV radiation (Rastogi et al., 2015), and survive extremely high temperatures (Inoue et al., 2001) and harsh conditions (Kasowska-Żok et al., 2014). Being able to perform oxygenic photosynthesis and (in part) nitrogen fixation, their growth in water ecosystems is usually limited by phosphorus availability. Increased P-loading caused by human activities is directly responsible for eutrophication of surface waters, causing uncontrolled *blooming* of several cyanobacterial species (Schindler, 1977). An increasing body of evidence suggests that cyanobacteria have evolved a versatile metabolic machinery for either the synthesis or the utilization of various forms of reduced phosphorus, including phosphonates (Benitez-Nelson, 2015; Cottingham et al., 2015; Van Mooy et al., 2015). However, the enzymological basis of this ability is still largely uncharacterized (Rivoal et al., 1998; Syiem and Bhattacharjee, 2014).

With respect to organophosphonates, the transport across the cell membrane seems to represent the limiting factor for their utilization (Forlani et al., 2008). This notwithstanding, it is now well established that the ability to degrade phosphonates is widespread among bacteria (Cook et al., 1978; Bujacz et al., 1995; Dyhrman et al., 2006; Lipok et al., 2007; Singh, 2009; Ford et al., 2010; Gomez-Garcia et al., 2011; Sviridov et al., 2015; Zhao et al., 2015). Interestingly, the pathway leading to the metabolization of the same compound may be completely different even in members of the same cyanobacterial genus. (Gomez-Garcia et al., 2011). The availability of the whole genome sequence for a remarkable number of cyanobacterial strains showed that they possess orthologs of

genes encoding enzymes involved in phosphonate breakdown, but also that a significant diversity is present. The *pho* regulon in *Nostoc* sp. PCC 7120 was found to consist in a single locus with at least three transcriptional units. Interestingly, it comprises an additional putative gene, non present in *E. coli*, which may encode a member of the two-histidine phosphodiesterase superfamily (Hove-Jensen et al., 2014). When the presence of the *pho* regulon was analysed in 19 sequenced cyanobacterial genomes, some genes that are directly involved in phosphorus assimilation were shown to not be under the regulation of the orthologue of PhoB in *E. coli*, the regulator SphR, which was not even present in three cyanobacterial genomes (Su et al., 2007)**.** However, the biochemical characterization of organophosphonate biodegradation processes by cyanobacteria is still scarce and incomplete. On the other hand, it has been shown that many enzymes in cell-free extracts from cyanobacterial cultures, although unrelated to phosphonate metabolization, retain their activity following freezing or lyophilization (Sanevas et al., 2006; Hao et al., 2008; Osswald et al., 2009; Wu et al., 2011).

In previous studies the cyanobacterium *Spirulina platensis* was found able to accomplish partial removal of a model polyphosphonate from wastewaters (Forlani et al., 2011; Forlani et al., 2013), but no information was obtained concerning the steps in the biodegradation process. Here we report the ability of a collection strain of *Anabaena variabilis* to use DTPMP as a source of phosphorus. *In vivo* characterization of phosphonate metabolism was hampered by low uptake rates at low concentrations, and cytotoxic effects at high levels. To address this problem, the formation of intermediates and products was investigated following the incubation of the polyphosphonate with cell-free extracts.

Results & Discussion

At millimolar concentrations the polyphosphonate DTPMP inhibits the proliferation of the cyanobacterium *Anabaena variabilis* in standard Bg11 medium, but at lower levels it enhances growth in a modified, P-deficient medium

Up to 0.1 mM, the addition of DTPMP to the culture medium of *Anabaena variabilis* strain CCALA 007 did not affect cyanobacterial growth. On the contrary, a significant increase of the mean growth rate was evident, which was paralleled by a corresponding decrease of doubling time (Table 1). Above this threshold, however, cell viability was rapidly and completely lost. Similar results have been already reported for various cyanobacterial species treated with other potentially toxic phosphonates. For instance, in the case of the non-selective herbicide glyphosate no cytotoxic effects were found up to 0.3 – 1 mM also for some strains that possess a glyphosate-sensitive target enzyme, such as *Microcystis aeruginosa* and *Leptolyngbya boriana*, but cell growth was completely abolished at slightly higher concentrations (Forlani et al., 2008). Some circumstantial evidence indeed strengthened the possibility that at micromolar concentrations glyphosate uptake proceeds through a high affinity phosphate transporter showing low affinity toward the phosphonate (Hetherington et al., 1998), allowing only low amounts of the herbicide to be incorporated into the cell. At millimolar levels, diffusion across the membrane does in contrast take place, causing the attainment of high intracellular concentrations, which in turn trigger cell death. Further evidence supporting a low permeability of polyphosphonates across the biological membranes was obtained with hexamethylenediamine-*N*,*N*,*N*′,*N*′-tetrakis(methylphosphonic acid) (Dequest® 2054), as the ability of the cyanobacterium *Spirulina platensis* to metabolize this xenobiotic was significantly improved by treatment of the cells with sublethal doses of detergent (Forlani et al., 2013). On the other hand, the stimulatory effect on growth at low concentrations is a well-known effect of phosphonates, and may be ascribed to their chelating properties, avoiding precipitation and allowing a better incorporation of inorganic nutrients from the culture medium (Forlani et al., 1997; Forlani et al., 2011).

Interestingly, when the same experiment was performed with a modified medium in which the inorganic P source had been omitted (Bg11-P), in the presence of the phosphonate after an early partial cell lysis the culture attained both a higher growth rate and increased final biomass (Fig. 2). A higher rate could depend on the initial release of Pi from cells underlying lysis, but this would not explain the higher final organic matter. On the other hand, negative controls in which the medium had been added with DTPMP and maintained under the same experimental conditions but without inoculation showed neglible spontaneous degradation of the polyphosphonate. Although indirect, this evidence therefore

suggested that *Anabaena variabilis* may possess the ability of catalysing an at least partial hydrolysis of this xenobiotic, leading to the release of significant amounts of inorganic phosphate that may sustain growth even in the absence of any other P source.

Table 1. Effect of increasing concentrations of DTPMP on the growth of *Anabaena variabilis.* Cyanobacterial cultures were started in standard (Bg11) liquid medium or in medium depleted of inorganic phosphate (Bg11-P), and growth was followed as the increase of chlorophyll content. Following logarithmic transformation, growth rates and generation doubling times (DT) were calculated from the linear part of each curve. For the former parameter, results were expressed as percent of rates for untreated controls in Bg11 medium, and are means ± SD over three independent replicates; n.c., not calculable.

Fig. 2. Time course of *Anabaena variabilis* growth in the presence of DTPMP. The polyphosphonate was added to either the standard Bg11 medium (left panel), or a modified medium (Bg11-P) in which potassium phosphate had been omitted (right panel). Growth was followed as the increase of chlorophyll content, and data were linearized by logarithmic transformation. Each point is the mean ± SD over three independent replications.

Crude extracts from *Anabaena* cells are able to catalyse degradation of DTPMP

To obtain a direct evidence supporting this hypothesis, ^{31}P NMR analysis may represent an effective tool, as it can be applied either to spent media or directly on living cells. Both approaches have been previously employed by our group to follow disappearance of millimolar amounts of the phosphonate herbicide glyphosate (Lipok et al., 2007; Forlani et al., 2008) or the polyphosphonate Dequest® 2054 (Forlani et al., 2011; Forlani et al., 2013) and the concomitant release of inorganic phosphate in biodegradation processes. However, in this case the treatment of cells with millimolar amounts of DTPMP would have caused a rapid loss of cell viability, and the optimal concentration of 0.1 mM would have been too low to be reliably detected with *in vivo* ^{31}P NMR measurements. As an alternative, the analysis of crude extracts from *Anabaena* cells following incubation for increasing time with 1 mM DTPMP was performed.

Due to the asymmetry of the molecule, ^{31}P NMR measurements immediately after the addition of the pure standard to cell extracts showed the presence of two peaks in the spectrum, with a peak area ratio of 1:4. The methylenephosphonic group located in the centre of the molecule, having a different chemical surrounding, gave a signal with a chemical shift (δ) of 16.5 ppm, whereas all the four external phosphonate moieties gave a signal at δ = 7.5 ppm (Fig. 3). Negative controls in which the polyphosphonate had been added to the extraction buffer and incubated under the same conditions did not show any apparent spontaneous degradation. On the contrary, when 1 mM DTPMP was incubated for 72 h at 37 °C with crude extracts from *Anabaena* cells that have been grown in standard Bg11 medium in the absence of the phosphonate, a sharp decrease of the signals coming from the substrate was evident, with the concomitant appearance of several other signals (Fig. 4). Six out of seven of these new peaks were identified by the addition of internal standards (Table 2). The signal at δ = 15.4 was tentatively assigned based on literature data (Hove-Jensen et al., 2011, 2012) to 5'-phospho-α-D-ribosyl-1'-(*N*-acetamidomethylephosphonatic acid). Indeed, the presence of phosphates bound to a ribose in the supernatant from *E. coli* cultures after incubation with some phosphonates has been reported (Hove-Jensen et al., 2011).

Fig. 3. ^{31}P NMR spectrum of *Anabaena variabilis* crude extracts immediately after the addition of 1 mM DTPMP. The signal labelled with "○" corresponds to phosphorus atoms located in outer *N,N'*-di(methylenephosphonic) moieties, whereas that labeled with "Δ" is the signal assigned to the inner *N*-methylenephosphonic group.

Fig. 4. ^{31}P NMR spectrum of *Anabaena variabilis.* crude extracts 72 h after the addition of 1mM DTPMP. The phosphorus peaks with chemical shifts close to 7.5 and 16.5 ppm were attributed to DTPMP, whereas the other peaks correspond to intermediates and products of its biotransformation.

Table 2. The phosphonate intermediates and by-products of DTPMP biodegradation, determined experimentally and basing on the reference analysis

An additional feature was the presence in the spectrum of several low signals in the region at δ = 2 – 4, characteristic of inorganic phosphate, suggesting complete mineralization of at least part of the substrate. Being these signals absent or much lower in parallel controls in which cell-free extracts had been incubated in the absence of the polyphosphonate, it seems more likely that these phosphates are produced by a phosphodiesterase similar to that encoded by the *phnP* gene (Hove-Jensen et al., 2011) than by unspecific phosphatases acting on some endogenous substrate. However, more data are required to shed light on these aspects.

The time course of incubation with cell-free extracts provides information about the sequence of the steps involved in DTPMP biotransformation

To obtain further details on the biotransformation process, the analysis was repeated on samples incubated for shorter times at 24 h intervals. The results are summarized in Fig. 5.

Fig. 5. Heat map showing the products of DTPMP breakdown by cell-free extracts from *Anabaena* cells. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to ^{31}P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively.

After 24 h of incubation the appearance of similar quantities of AMPA, AcAMPA and MPA suggested that all the five MP groups in the DTPMP molecule are metabolized at a similar rate. The presence of *N*-acetylaminomethylphosphonic acid (δ = 14.2 ppm) was quite unexpected, and could be hypothetically attributed to the action of PhnO, an aminoalkylphosphonate *N*-acetyltransferase (Hove-Jensen et al., 2014). *N*-acetylation prior of C-P bond cleavage can be required in order to eliminate its charge and ensure the stability of the intermediate (Hove-Jensen et al., 2012). MPA (δ = 21.9 ppm)

and AMPA may also be considered as intermediates in the conversion of DTPMP to phosphates (Kamat et al., 2011). Interestingly, the amount of inorganic phosphorus released was only about 33% of that calculated from the difference between the DTPMP used and the sum of the intermediates produced. This suggests that part of the phosphate resulting from the biotransformation process could be incorporated *in vitro* into some other organic compounds (Hove-Jensen et al., 2012). Moreover, the ratio among the observed products suggest that the cleavage of the C-N bond at the central nitrogen atom of DTPMP precedes that on terminal nitrogen atoms of the chain. Similarly, in the case of microbial degradation of some methylamines, including long-chain quaternary ammonium compounds, tetramethylammonium chloride and nitrilotriacetic acid, the initial cleavage of the C–N bond was found to be required to allow the access to the alkyl chain (Van Ginkel et al., 1992; Van Ginkel, 1996; Mohamed Ahmed et al., 2009).

MPA was detected also following 48 h of incubation, concomitantly with the identification of MAMPA (δ = 13.5 ppm), whose concentration remained relatively constant thereafter. Due to its chemical structure, MAMPA is barely accessible to the enzymes, and thus does not undergo acetylation. The proposed mechanism is supported also by the change of the ratio between the area of the two signals coming from DTPMP: from 24 to 48 h incubation, that at 7.5 ppm decreased by 46%, whereas the signal related to the central phosphonic group at 16.5 ppm was reduced only by 12%.

After 72 h, the hydrolysis of the phosphonic moiety anchored to the central nitrogen atom seems to have proceeded further (Fig. 5). At the same time an additional release of AMPA and the increase in the levels of AcAMPA strengthen the hypothetical involvement of PhnO. Interestingly, a new product, namely iminobis(methylenephosphonic) acid (IBMPA) (δ = 8.4 ppm), was detected, albeit in relatively low amounts. The appearance of the signal for IBMPA was paralleled by a proportional decrease of those related to the outer phosphonic groups, confirming that the cleavage of C-N bonds next to external nitrogen atoms proceeds with very low efficiency.

Overall, these results suggested that in *Anabaena* cell-free extracts DTPMP biotransformation may proceed through multiple pathways, and allowed us to propose the sequential process shown in Fig. 6. The suggested mechanisms seem at least in part consistent with analogous routes previously described for *E. coli*. In this enterobacterium, alkylphosphonic acids initially react with an ATP

molecule; the product is then dephosphorylated, with the release of pyrophosphate and an intermediate that in turn undergoes cyclization, yielding a 5'-phospho-α-D-ribosyl 1,2-cyclic phosphonate. The latter is further metabolized by other hydrolases, leading to the final release of diphosphate and phosphate ions (Mazumder, 2002; Kononova et al., 2007; Kamat et al., 2011; Hove-Jensen et al., 2012; Hove-Jensen et al., 2014). Although the presence of ribosyl intermediates in *A. variabilis* extracts was not definitely demonstrated due to their presumably low concentrations and the unavailability of pure standard, the peak at 15.4 ppm may be most likely attributed to 5'-phospho-α-D-ribosyl 1'-(*N*-acetylaminomethylephosphonic acid).

Fig. 6. Proposed pathways for the breakdown of the polyphosphonate DTPMP in crude extracts from *Anabaena variabilis*. Lowercase letters emphasize the bond whose hydrolysis leads to the release of a given intermediate. Compounds: 1. Aminomethylphosphonic acid, 2. (*N*-acetylaminomethylphosphonic acid , 3. 5'-triphospho-α-D-ribosyl 1'-(*N*-acetamidomethylphosphonate), 4. 5'-phospho-α-D-ribosyl 1'-(*N*-acetylaminomethylphosphonic acid), 5. 5'-phospho-α-D-ribosyl 1,2-cyclic phosphonate, 6. α-D-ribosyl 1,5-bisphosphonate, 7. 5-phospho-α-D-ribosyl 1-diphosphonate, 8. diphosphate ion (PP_i), 9. phosphate ion (P_i), 10. methylphosphonic acid, 11. *N*-(methylamino)bis(methylenephosphonic acid), 12. (*N*-methylamino)methylphosphonic acid, 13. iminobis(methylenephosphonic) acid.

To further strengthen the proposed pathway, similar experiments were carried out by incubating extracts from *A. variabilis* cells with some of the intermediates that had been identified after incubation with DTPMP, provided singly. The results, presented in Supplemental Figures 1A-E, were on the whole consistent with the scheme outlined in Fig. 6. Indeed, AMPA underwent acetylation prior to be converted to 5'-phospho-α-D-ribosyl 1'-(*N*-acetylaminomethylephosphonic acid) and cleaved, being AcAMPA utilization apparently more rapid than its production (Supplemental Fig. 1A). When AcAMPA was directly added, the same was evident (Supplemental Fig. 1B). Under the experimental conditions employed, it appeared that MAMPA was not metabolized (Supplemental Fig. 1E). Following the incubation of extracts with MABMP, the signals for MAMPA and IBMPA were in fact detected (Supplemental Fig. 1D). Interestingly, in this case also inorganic phosphorus forms were detectable: since MAMPA is not hydrolyzed, this implies that IBMPA could be further metabolized

279 *via* some unknown reactions. This was confirmed when IBMPA was the substrate (Supplemental Fig.
280 1F). Even more interestingly, when crude extracts were incubated with MPA, no metabolization
281 products were evident (Supplemental Fig. 1C). Although it cannot be excluded that this may depend
282 on either the lack of some co-factor or the lability of the enzyme(s) responsible for the reaction, and
283 that the latter can take place *in vivo*, this suggest a different system than in *E. coli*, where MPA is
284 efficiently degraded by means of the C-P lyase complex (Kononova and Nesmeyanova, 2002).
285
286 **DTPMP metabolism in *Anabaena variabilis* does not depend, but is influenced by the**
287 **phosphate status of the cell**
288 Cell-free extracts were found able to catalyse an almost quantitative hydrolysis of DTPMP, as
289 its initial level decreased by 30% after 24 h of incubation, and by 71-75% after 72 h, a result that
290 further strengthens the possibility that a low uptake rate may represent the limiting step for
291 phosphonate metabolism by cyanobacteria. It is also worth of notice that these results were obtained
292 with extracts from *Anabaena* cells grown in the presence of inorganic phosphate and in the absence of
293 the polyphosphonate.
294 To obtain more information on the mechanisms possibly regulating the expression of the
295 enzymes responsible for DTPMP breakdown, the experiment was repeated with cell-free extracts
296 prepared from cells grown in the presence or in the absence of the substrate, and under conditions of
297 inorganic phosphate availability or starvation. The results obtained after 48 h of incubation of these
298 extracts with 1 mM DTPMP are shown in Fig. 7. In all cases in which cyanobacterial cells had been
299 cultured in the presence of the phosphonate or had been subjected to P deprivation, the consumption of
300 the substrate increased by about 50%.
301 Concerning the appearance of intermediates in DTPMP breakdown, the same
302 organophosphorus compounds that had been previously found with extracts prepared from cells grown
303 in standard medium were evident in ^{31}P NMR spectra (Fig. 8). The main difference consisted in the
304 lack of detectable AMPA in extracts from cells grown in Bg11-P and Bg11+DTPMP. Being the
305 activation of completely different pathways unlikely, this result suggests a faster transformation of

AMPA into AcAMPA, further emphasising the importance of acylation of the cleaved fragments in phosphonate matabolism. Overall, the highest efficiency of biodegradation, as judged from the release of inorganic forms of phosphorus, was obtained with extracts from cells grown in the presence of DTPMP as the only P-source.

Fig. 7. Effects of cell growth conditions on the rate of DTPMP metabolism by *Anabaena* cell-free extracts. Cultures were grown for 2 weeks in liquid media either containing or not inorganic phosphate and the polyphosphonate in all four possible combinations, as indicated. Extracts prepared from the same amount of cells (1 g fresh weight) were incubated for 48 h at 37 °C with 1 mM DTPMP, then the residual concentration of the substrate was determined by ^{31}P NMR. Data were expressed as percent decrease of the area of both signals (at 7.5 and 16.5 ppm) that originate from the phosphonate groups in the DTPMP molecule, and are mean ± SD over three replicates.

Fig. 8. Heat map showing the products of phosphonate breakdown by cell-free extracts prepared from *Anabaena* cells grown in the presence or in the absence of inorganic Pi and DTPMP. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Panels refer to ^{31}P NMR analysis carried out following 48 h-incubation of 1 mM DTPMP at 37 °C with extracts prepared from 1 g cells (fresh weight) grown in Bg11, Bg11+DTPMP, Bg11-P and Bg11-P+DTPMP, as indicated.

Conclusions

The results obtained in this work clearly demonstrated the ability of the cyanobacterium *Anabaena variabilis* to catalyse almost complete mineralization of the polyphosphonate DTPMP. This capability seems limited *in vivo* by a low uptake, yet the substrate sustained cyanobacterial growth when provided at low levels as the only source of phosphorus. This notwithstanding, DTPMP at millimolar concentration caused a complete loss of cell viability. Its toxicity may depend on the presence of the five strongly hydrophilic aminophosphonic groups, that could either have an impact on

cell membrane functioning (Forlani et al., 2013), or interfere with the incorporation of some essential mineral nutrient through their chelating properties (Forlani et al., 1997). In any case, cell-free extracts from *Anabaena* cells were found to efficiently convert the phosphonate molecule into a complex series of P-containing intermediates. The analysis by ^{31}P NMR at increasing time following the addition of the substrate allowed us to hypothesize on the sequential steps in DTPMP breakdown.

The enzymological bases of DTPMP utilization are still to be elucidated in detail. However, some hints were obtained from the detection of AcAMPA in the reaction mixture. This compound in *E. coli* is an obligatory intermediate in the decomposition of AMPA by C-P lyase (Hove-Jensen et al., 2012), and its presence supports a main role for an aminoalkylphosphonate *N*-acetyltransferase (Kaneko, 2001; Hove-Jensen et al., 2014; Teikari et al., 2015). However, the involvement of a C-P lyase in *Anabaena variabilis* is questionable, since the activity of this multienzymatic complex is usually lost following membrane solubilization (Ternan et al., 1998; Hove-Jensen et al., 2014), and its expression would require P-deprivation (Wackett et al., 1987; Ternan et al., 1998). Moreover, DTPMP metabolism in *Anabaena variabilis* gave rise to the formation of other phosphonates, namely IBMPA and MABMP, which most probably arise as the result of different biotransformation pathway(s), and MPA is apparently not metabolized. MABMP seems to be converted in turn into its *N*-methyl derivative – MAMPA, which cannot be further metabolized. In fact, *N*-methylation is a widely adopted reaction for detoxification of xenobiotics by living cells (McQueen, 2010). Although their direct utilization may be hampered by low uptake rates, understanding of the metabolic basis of cyanobacterial utilization of polyphosphonates would open new and interesting perspectives toward the development of biological processes for the bioremediation of these pollutants.

Experimental Procedures

Materials

Anabaena variabilis Kutzing strain CCALA 007, isonym of *A. variabilis* ATCC 29413 and *Nostoc sp.* PCC 7937, was obtained from the Culture Collection of Autotrophic Organisms at the Institute of Botany of the Academy of Sciences of the Czech Republic. Unless specified otherwise,

chemicals were purchased from POCh S.A. (Gliwice, Poland). DTPMP was obtained from Zschimmer & Schwarz GmbH & Co KG (Mohsdorf, Germany) in the frame of a scientific cooperation.

Cyanobacterial growth

A. variabilis was routinely grown at 25 ± 1° C under 16 h : 8 h day : night at 200 µmol m^{-2} s^{-1} PAR in 250 mL Erlenmeyer flasks containing 60 mL of Bg11 medium (ATCC 616) (Allen, 1968; Ripka, 1979). Cultures were revitalized every 14 days by transferring 10 mL aliquots to 50 ml fresh medium. For experiments, cultures were started by transferring a suitable volume of a stock culture to fresh Bg11 medium so as to obtain an initial concentration of 1 mg L^{-1} chlorophyll. Chlorophyll content was measured in metanolic extracts, as previously described (Lipok et al., 2010).

To assess the effect of DTPMP on cyanobacterial growth, parallel cultures were set up in Bg11 medium supplemented or not with the phosphonate at concentrations ranging from 0.05 to 0.5 mM. To verify the effect of P_i depletion, the same set of treatments were carried out with a modified medium in which K_2HPO_4 had been omitted (Bg11-P); in this case potassium was provided as KNO_3 (34 mg L^{-1}), and $NaNO_3$ was reduced to 1.47 g L^{-1}. Growth was followed for two weeks as the increase of chlorophyll concentration. Following logarithmic transformation, data were used to calculate growth rate constants and generation times. Data were expressed as percent of untreated controls in standard medium, and are mean ± SD over three replicates.

Preparation of cell-free extracts

Cells were extracted by a modification of a previously published protocol (Forlani et al., 2015). Briefly, *Anabaena* cultures in the late exponential phase of growth were harvested by centrifugation for 5 min at 3000 *g* at 4°C. Pelleted material was frozen at -24°C for 45 min, then transferred to a pre-cooled mortar and immediately ground with 2 g g^{-1} Al_2O_3. The homogenate was resuspended in 10 mL g^{-1} of 100 mM Tris-HCl buffer (Sigma, USA), pH 7.2, containing 250 mM NaCl (extraction buffer). Following centrifugation at 5000 *g* at 4°C for 10 min, the supernatant was added with solid ammonium sulphate so as to obtain 70% saturation. Salted-out proteins were pelleted by centrifugation as above, and finally resuspended in a minimal volume of extraction buffer.

390

391 **NMR analysis**

392 Aliquots (0.5 mL) of cell-free extracts were sterilized by filtration (0.22 µm), transferred to
393 sterile NMR tubes and added with DTPMP to a final concentration of 1.0 mM. Exact blanks were
394 carried out with extraction buffer, and negative controls were performed with extracts not added with
395 the phosphonate. Tubes were incubated at 37°C in the dark for increasing time (24, 48 and 72 h), and
396 then subjected to ^{31}P NMR analysis, which was performed using a Bruker Avance DRX 400
397 spectrometer operating at 161.976 MHz. The data were acquired at 20 ± 1 °C, using a 30° pulse, a
398 1.337 s acquisition time and a 0.5 s relaxation delay, with 20 Hz spinning (5-mm probe). The number
399 of scans was 900, with FID resolution of 0.374 Hz. A 10 mM solution of H_3PO_4 was used as the
400 internal reference standard.

401 To determine the corresponding chemical shifts and identify potential products of DTPMP
402 metabolism, stock solutions of standards were prepared at a concentration of 1 mg mL^{-1} in extraction
403 buffer. Aliquots (25 µL) of these solution were added just before the analysis to NMR tubes already
404 containing the cyanobacterial extract that had been incubated for a given time.

405 A semiquantitative estimate of the concentration of a given compound in these samples was
406 obtained by integrating the area of the signal with respect to that of the internal reference standard
407 using the software MestReNova (version 6.0.2, Mestrelab Research, Santiago de Compostela, Spain).
408 Data were further normalized and expressed *per* gram of cells (fresh weight) from which extracts had
409 been prepared.

410

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416

Conflict of Interest Statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table and Figures legends

Table 1. Effect of increasing concentrations of DTPMP on the growth of *Anabaena variabilis.* Cyanobacterial cultures were started in standard (Bg11) liquid medium or in medium depleted of inorganic phosphate (Bg11-P), and growth was followed as the increase of chlorophyll content. Following logarithmic transformation, growth rates and generation doubling times (DT) were calculated from the linear part of each curve. For the former parameter, results were expressed as percent of rates for untreated controls in Bg11 medium, and are means ± SD over three independent replicates; n.c., not calculable.

Table 2. The phosphonate intermediates and by-products of DTPMP biodegradation, determined experimentally and basing on the reference analysis

Fig. 1. Structure of the polyphosphonate diethylenetriaminepenta(methylenephosphonic) acid – DTPMP.

Fig. 2. Time course of *Anabaena variabilis* growth in the presence of DTPMP. The polyphosphonate was added to either the standard Bg11 medium (left panel), or a modified medium (Bg11-P) in which potassium phosphate had been omitted (right panel). Growth was followed as the increase of chlorophyll content, and data were linearized by logarithmic transformation. Each point is the mean ± SD over three independent replications.

Fig. 3. ^{31}P NMR spectrum of *Anabaena variabilis* crude extracts immediately after the addition of 1 mM DTPMP. The signal labelled with "○" corresponds to phosphorus atoms located in outer *N,N'*-di(methylenephosphonic) moieties, whereas that labeled with "Δ" is the signal assigned to the inner *N*-methylenephosphonic group.

Fig. 4. ^{31}P NMR spectrum of *Anabaena variabilis* crude extracts 72 h after the addition of 1mM DTPMP. The phosphorus peaks with chemical shifts close to 7.5 and 16.5 ppm were attributed to DTPMP, whereas the other peaks correspond to intermediates and products of its biotransformation.

Fig. 5. Heat map showing the products of DTPMP breakdown by cell-free extracts from *Anabaena* cells. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to ^{31}P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively.

Fig. 6. Proposed pathways for the breakdown of the polyphosphonate DTPMP in crude extracts from *Anabaena variabilis*. Lowercase letters emphasize the bond whose hydrolysis leads to the release of a given intermediate. Compounds: 1. Aminomethylphosphonic acid, 2. (*N*-acetylaminomethylphosphonic acid , 3. 5'-triphospho-α-D-ribosyl 1'-(*N*-acetamidomethylphosphonate), 4. 5'-phospho-α-D-ribosyl 1'-(*N*-acetylaminomethylphosphonic acid), 5. 5'-phospho-α-D-ribosyl 1,2-cyclic phosphonate, 6. α-D-ribosyl 1,5-bisphosphonate, 7. 5-phospho-α-D-ribosyl 1-diphosphonate, 8. diphosphate ion (PP_i), 9. phosphate ion (P_i), 10. methylphosphonic acid, 11. *N*-(methylamino)bis(methylenephosphonic acid), 12. (*N*-methylamino)methylphosphonic acid, 13. iminobis(methylenephosphonic) acid.

Fig. 7. Effects of cell growth conditions on the rate of DTPMP metabolism by *Anabaena* cell-free extracts. Cultures were grown for 2 weeks in liquid media either containing or not inorganic phosphate and the polyphosphonate in all four possible combinations, as indicated. Extracts prepared from the same amount of cells (1 g fresh weight) were incubated for 48 h at 37 °C with 1 mM DTPMP, then the residual concentration of the substrate was determined by ^{31}P NMR. Data were expressed as percent decrease of the area of both signals (at 7.5 and 16.5 ppm) that originate from the phosphonate groups in the DTPMP molecule, and are mean ± SD over three replicates.

Fig. 8. Heat map showing the products of phosphonate breakdown by cell-free extracts prepared from *Anabaena* cells grown in the presence or in the absence of inorganic Pi and DTPMP. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Panels refer to ^{31}P NMR analysis carried out following 48 h-incubation of 1 mM DTPMP at 37 °C with extracts prepared from 1 g cells (fresh weight) grown in Bg11, Bg11+DTPMP, Bg11-P and Bg11-P+DTPMP, as indicated.

Fig. S1A. Heat map showing AMPA breakdown by cell-free extracts from *Anabaena* cells, and the comparison of intermediates and products. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to 31P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively.

Fig. S1B. Heat map showing AcAMPA breakdown by cell-free extracts from *Anabaena* cells, and the comparison of intermediates and products. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to 31P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively.

Fig. S1C. Heat map showing MPA breakdown by cell-free extracts from *Anabaena* cells, and the comparison of intermediates and products. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to 31P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively. Results suggest that no metabolization occurs under the experimental conditions used.

Fig. S1D. Heat map showing MABMP breakdown by cell-free extracts from *Anabaena* cells, and the comparison of intermediates and products. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to 31P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively.

Fig. S1E. Heat map showing MAMPA breakdown by cell-free extracts from *Anabaena* cells, and the comparison of intermediates and products. A semi-quantitative estimation of the concentration of a given

compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to 31P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively. Results suggest that no metabolization occurs under the experimental conditions used.

Fig. S1F. Heat map showing IBMPA breakdown by cell-free extracts from *Anabaena* cells, and the comparison of intermediates and products. A semi-quantitative estimation of the concentration of a given compound was obtained by normalizing the area of the corresponding peak with respect to that of the internal standard [0.01 M phosphoric acid (V)]. Upper, medium and lower panels refer to 31P NMR analysis carried out following 24, 48 and 72 h of incubation, respectively.

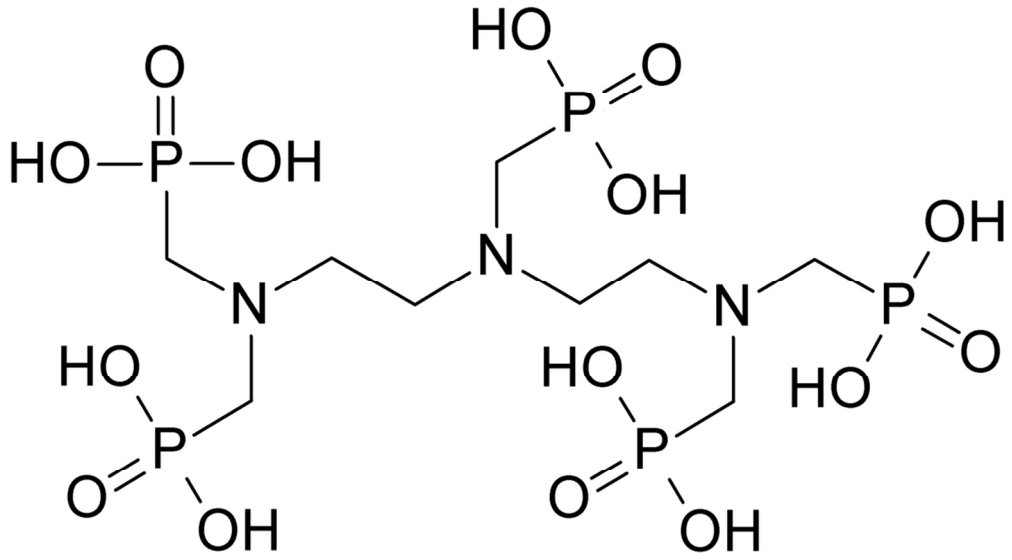

Fig. 1. Structure of the polyphosphonate diethylenetriaminepenta(methylenephosphonic) acid – DTPMP.
Fig. 1.
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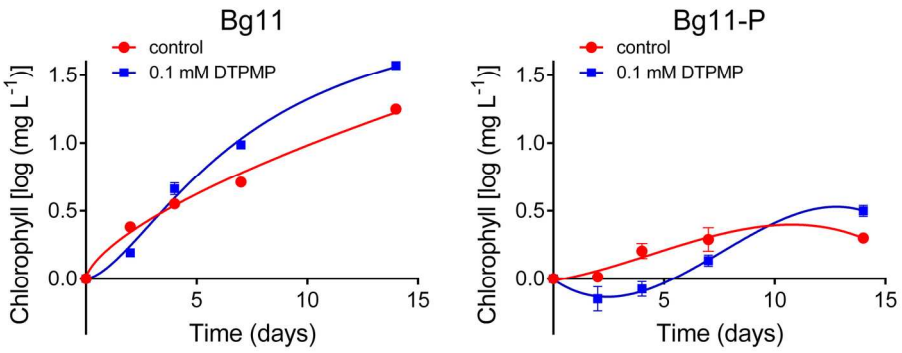

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Fig. 2.
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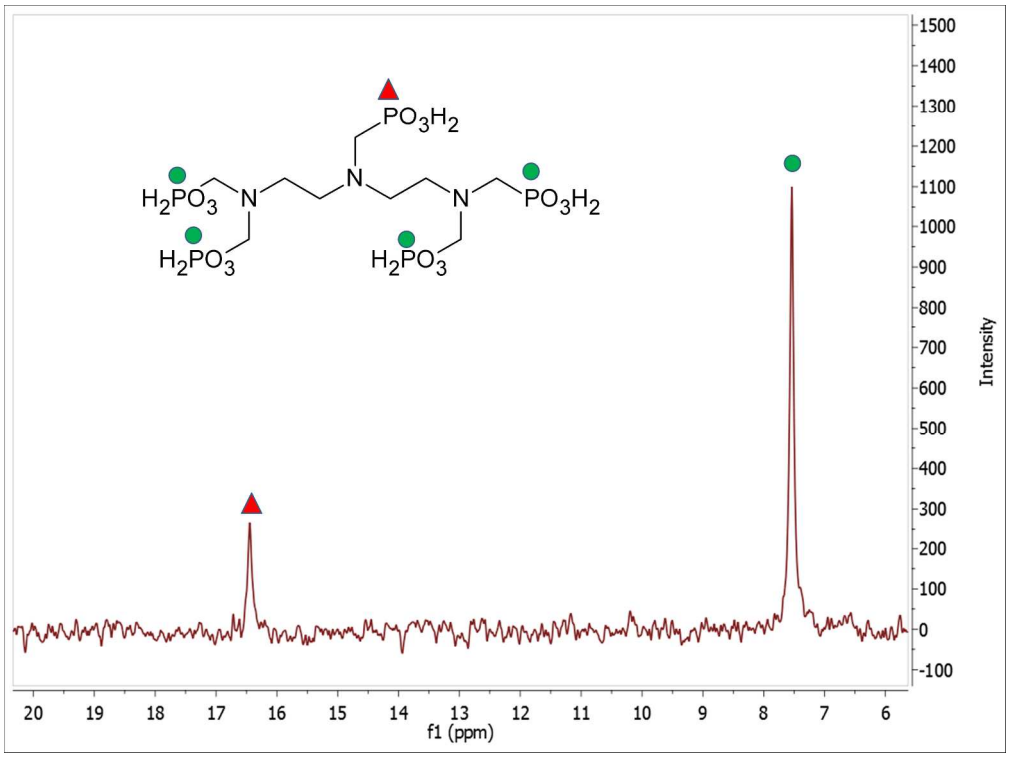

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Fig. 3.
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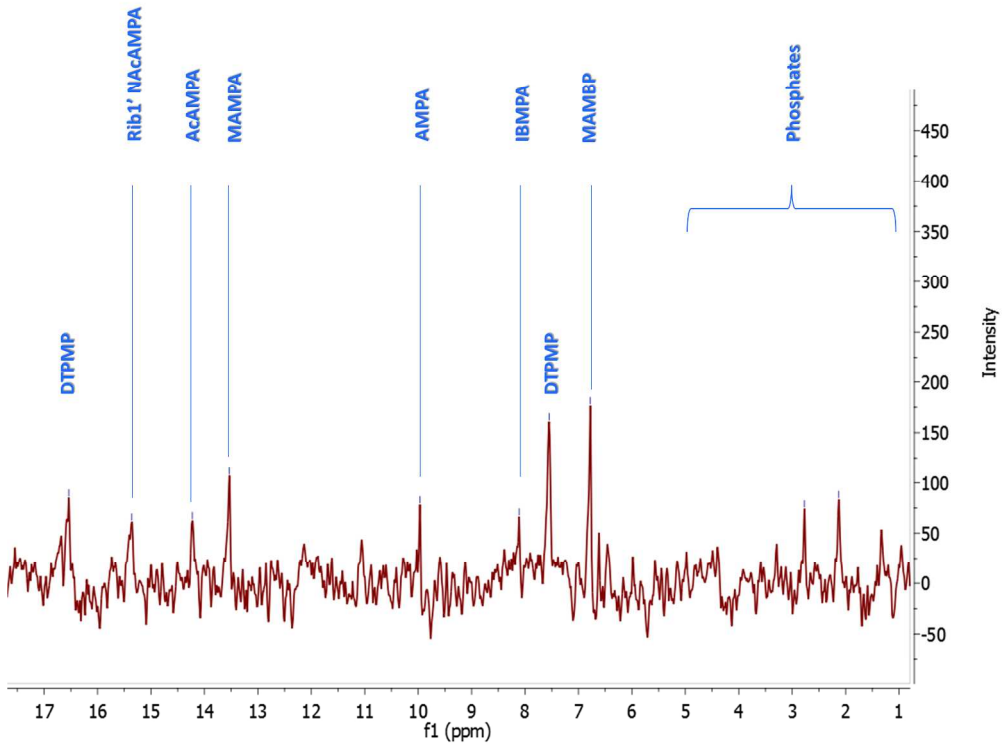

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Fig. 4.
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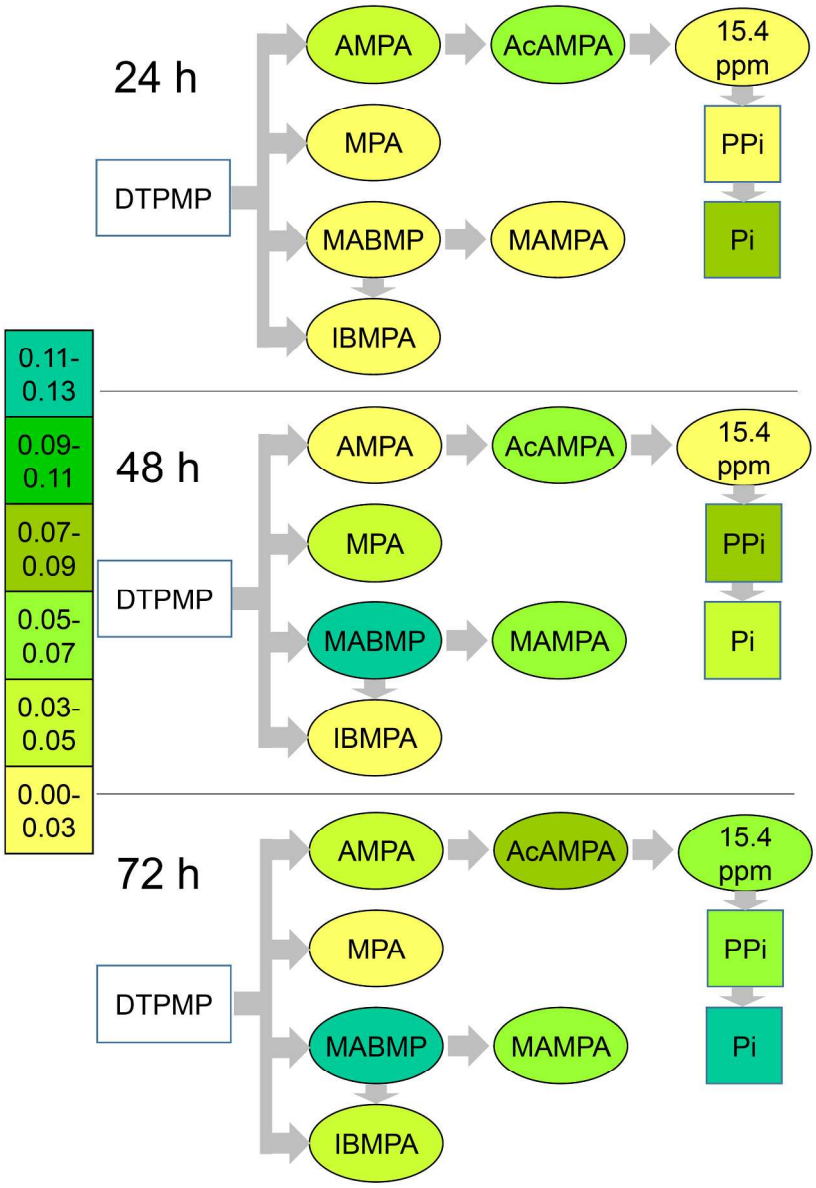

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Fig. 5.
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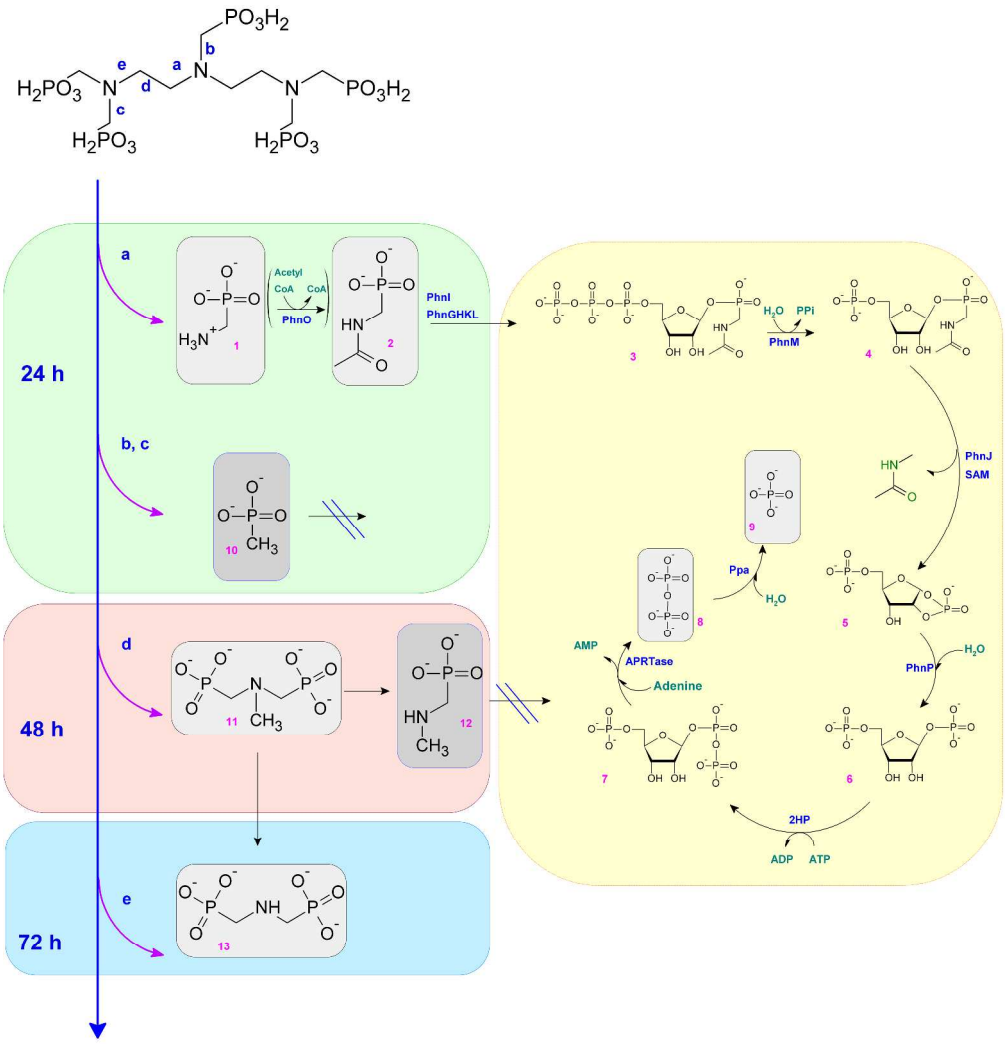

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Fig. 6.

411x428mm (300 x 300 DPI)

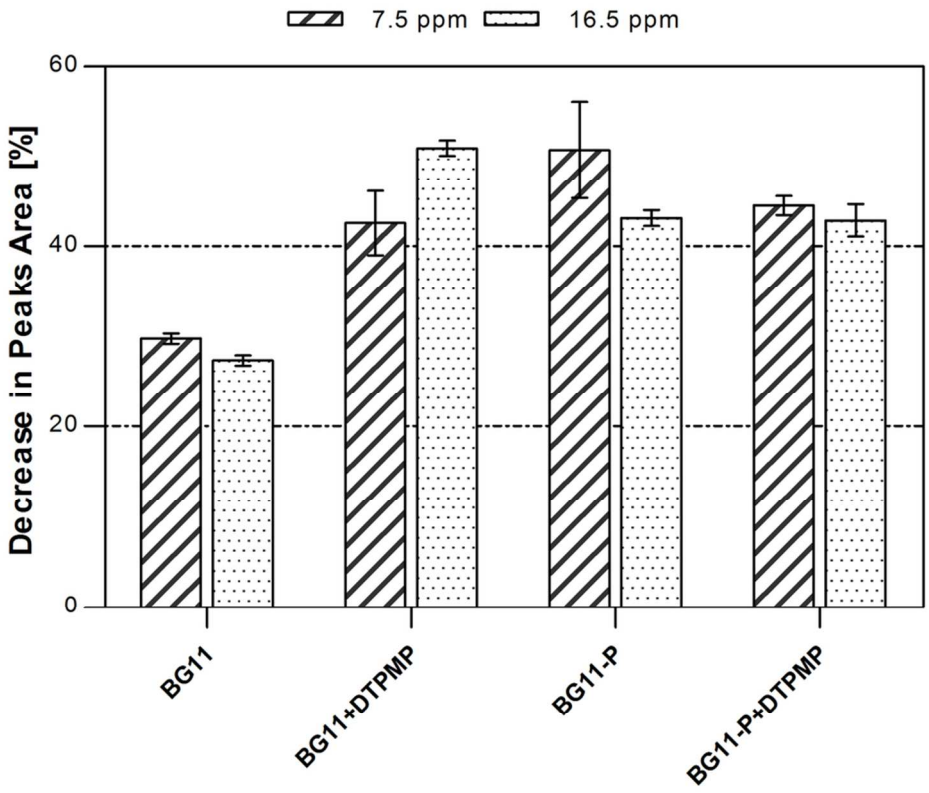

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Fig. 7.
86x73mm (300 x 300 DPI)

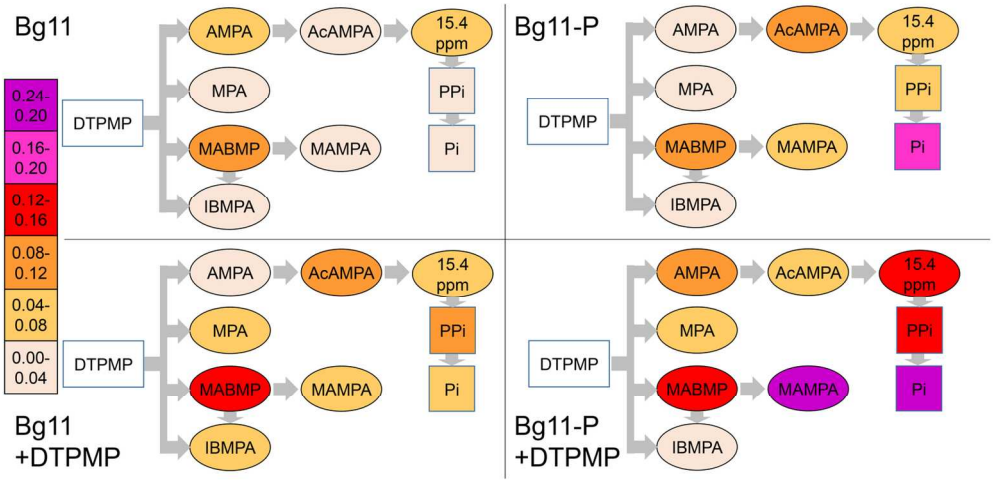

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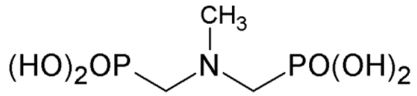
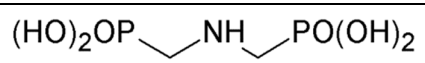
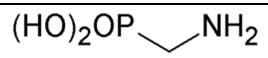
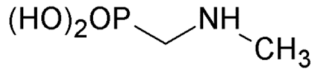
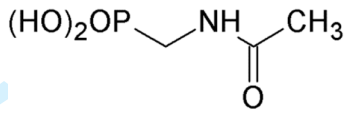
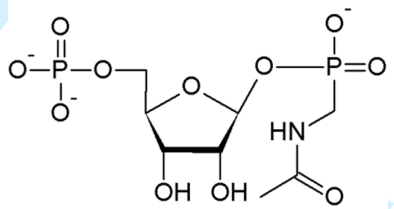
Fig. 8
138x68mm (300 x 300 DPI)

Table 1.

	DTPMP concentration (mM)	0.0	0.05	0.1	0.25	0.5
Bg11	DT [days]	3.38 ± 0.02	2.82 ± 0.05	2.69 ± 0.03	n.c.	n.c.
	Growth rate [%]	100 ± 2	118 ± 1	140 ± 3	0 ± 0	0 ± 0
Bg11-P	DT [days]	12.44 ± 0.51	11.93 ± 0.1	10.51 ± 1.14	n.c.	n.c.
	Growth rate [%]	27 ± 5	32 ± 1	44 ± 0	0 ± 0	0 ± 0

Table 2.

Abbrev.	Name	Chemical shifts [ppm]	Structure
MABMP	(*N*-methylamino) bis(methylenephosphonic) acid	δ = 6.6	
IBMPA	iminobis(methylenephosphonic) acid	δ = 8.4	
AMPA	aminomethylphosphonic acid	δ = 10.0	
MAMPA	(*N*-methylamino)methylphosphonic acid	δ = 13.5	
AcAMPA	(*N*-acetylmino)methylphosphonic acid	δ = 14.2	
Rib1' NAcAMPA	5'-phospho-α-D-ribosyl 1'-(*N*-acetylaminomethylephosphonatic acid)	δ = 15.4	
MPA	methylphosphonic acid	δ = 21.9