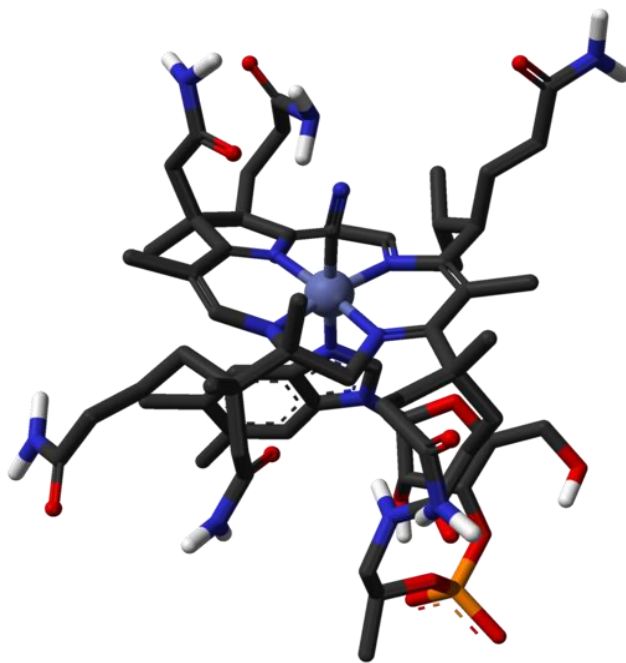


# DESY Summer Student Program 2010

## Report

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Trying to understand the B12 photo-reduction mechanism by synchrotron X-ray diffraction



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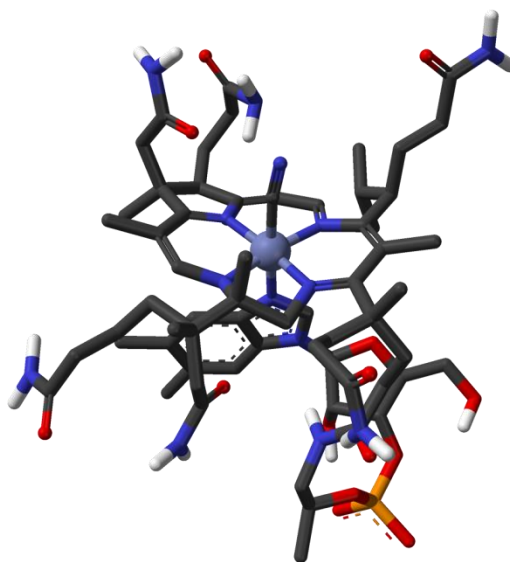
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## 1 Introduction

Vitamin B12, also called cobalamin, is a water soluble vitamin playing a key role in the normal functioning of the brain and nervous system, and for the formation of blood. It is normally involved in the metabolism of every cell of the human body, especially affecting DNA synthesis and regulation, but also fatty acid synthesis and energy production. As the largest and most structurally complicated vitamin, it can be produced industrially only through bacterial fermentation-synthesis.

A common synthetic form of the vitamin, cyanocobalamin (see fig. 1 – structure of the B12), does not occur in nature, but is used in many pharmaceuticals and supplements, and as a food additive, due to its stability and lower cost. In the body it is converted to the physiological forms, methylcobalamin and adenosylcobalamin, leaving behind the cyanide, albeit in minimal concentration.



**Fig. 1** Chemical structure of cyanocobalamin

Cyanocobalamin is known for its sensitivity to light of short-wavelengths [1] that causes reduction of the  $\text{Co}^{3+}$  metal center. Understanding of the photo-reduction mechanism is one of the challenging tasks for the photochemists. According to literature Hydrogen atoms play a key role in the photo-reduction process [2]. Nowadays one of the most prospective ways to find this out is X-ray crystallography [4]. Synchrotron X-ray diffraction allows obtaining diffraction pattern with very high resolution. This allows a very accurate determination of the crystallographic structure and the atomic bond distances by specific software (for example XDS and SHELX).

Therefore aims of the current work were:

- Investigation of the photo-reduction mechanism of vitamin B12
- Understanding whether photo reduction caused by a laser (402 and 532 nm) is similar to the one by synchrotron X-ray (0.8 Å) radiation

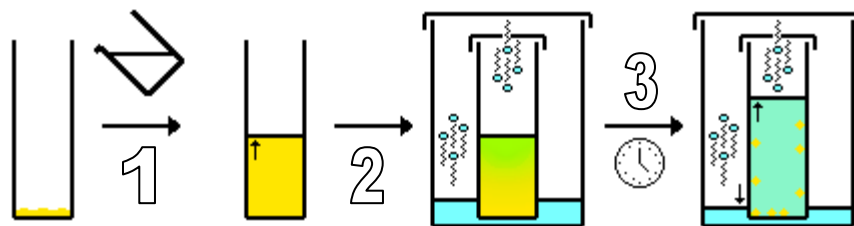
## 2 Experimental part

### 2.1 B12 single-crystal growth

High resolution structure determinations require diffraction pattern from single crystals of very high quality [5]. There are several synthetic routes to produce ‘perfect’ single crystals for X-ray crystallography:

- *Slow evaporation of a single solvent system* - typically the compound is dissolved in a suitable solvent and the solvent is allowed to slowly evaporate. Once the solution is oversaturated crystals can form.
- *Slow evaporation of a multi-solvent system* - the same as above, however as the solvent composition changes due to evaporation of the more volatile solvent. The compound is more soluble in the volatile solvent, and so the compound becomes increasingly insoluble in solution and crystallizes.
- *Slow diffusion* - a second solvent is allowed to evaporate from one container into a container holding the compound solution (gas-diffusion). As the solvent composition changes due to an increase in solvent that has gas-diffused into solution, the compound becomes increasingly insoluble in solution and crystallizes.
- *Interface/slow mixing* - two solvents mix (diffuse) by liquid-liquid diffusion. Typically a second solvent is "layered" carefully on top of the solution containing the compound. Over time the two solutions mix. As the solvent composition changes due to diffusion, the compound becomes increasingly insoluble in solution and crystallizes, usually at the interface.

In the current work slow diffusion of multi-solvent system was used. The principal schematic of the process is shown on the Fig. 2.



**Fig. 2** *Slow diffusion in multi-solvent system*

### Sequence of actions:

- 1) Solvent is added (clear) to the compound of interest (orange) in the first vessel yielding a solution (orange)
- 2) The vessel containing the solution is placed in a second vessel which contains a different solvent (blue). The outer vessel is sealed against atmosphere. The inner vessel is also sealed, although a small hole in the inner vessel is present. This hole allows solvent vapor (blue) to slowly evaporate from the outer vessel into the inner vessel yielding a mixed solvent system (green)
- 3) With time crystals (orange) appear from this saturated mixed solvent system (green-blue).

For the cobalamin single-crystal preparation the following crystallization procedure was used:

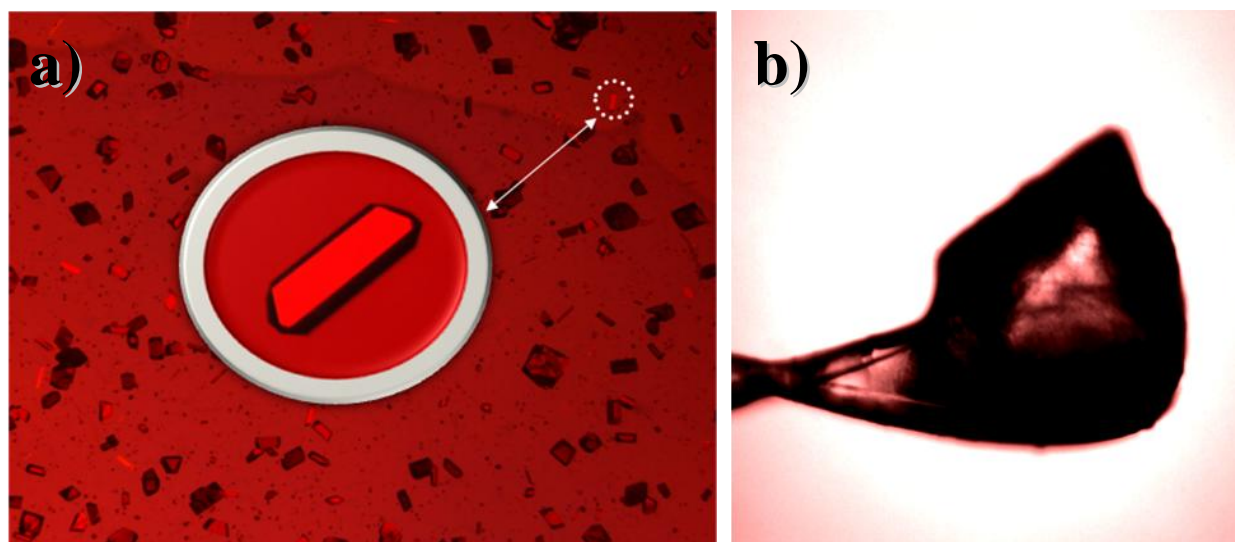
- We added 1ml of H<sub>2</sub>O to 12 mg of powdered B12 in a small eppendorf tube and shook it until it was completely dissolved. This mixture was then transferred into a Petri dish and iso-propanol was added in different ratios. After adding iso-propanol to the Petri dish it was put into an outer vessel containing pure iso-propanol. In order to prevent the evaporation of iso-propanol the outer vessel was sealed. Crystallization of proper (big enough and not polycrystalline) crystals took about 4 days. Different experiments were set up in order to find the optimal H<sub>2</sub>O: i-PrOH ratio to obtain B12 single-crystals of highest quality. It was found that the best ratios were 1:1.75 and 1:1.5.
- In addition an attempt to prevent photo reduction of B12 by daylight during crystallization was performed. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) oxidizing properties. 1 µl and 10 µl of a 30% aqueous solution of H<sub>2</sub>O<sub>2</sub> were added to some of the crystallization solutions.
- In order to check any possible effect of day light during crystal growth crystals were grown in two groups, one exposed to day light, the other one in the dark.

## 2.2 Laser radiation of the crystals

### 2.2.1 Sample preparation

#### 2.2.1.1 Fishing and mounting of the crystals

Diffraction experiments require specific crystal mounting techniques. B12 crystals grown in our experiments contain iso-propanol molecules, which tend to evaporate at ambient conditions thereby destroying the crystalline order. Therefore it's very important to omit evaporation of the solvent during the mounting process. All operations with the B12 crystals were performed under a cold nitrogen flow or in liquid nitrogen.

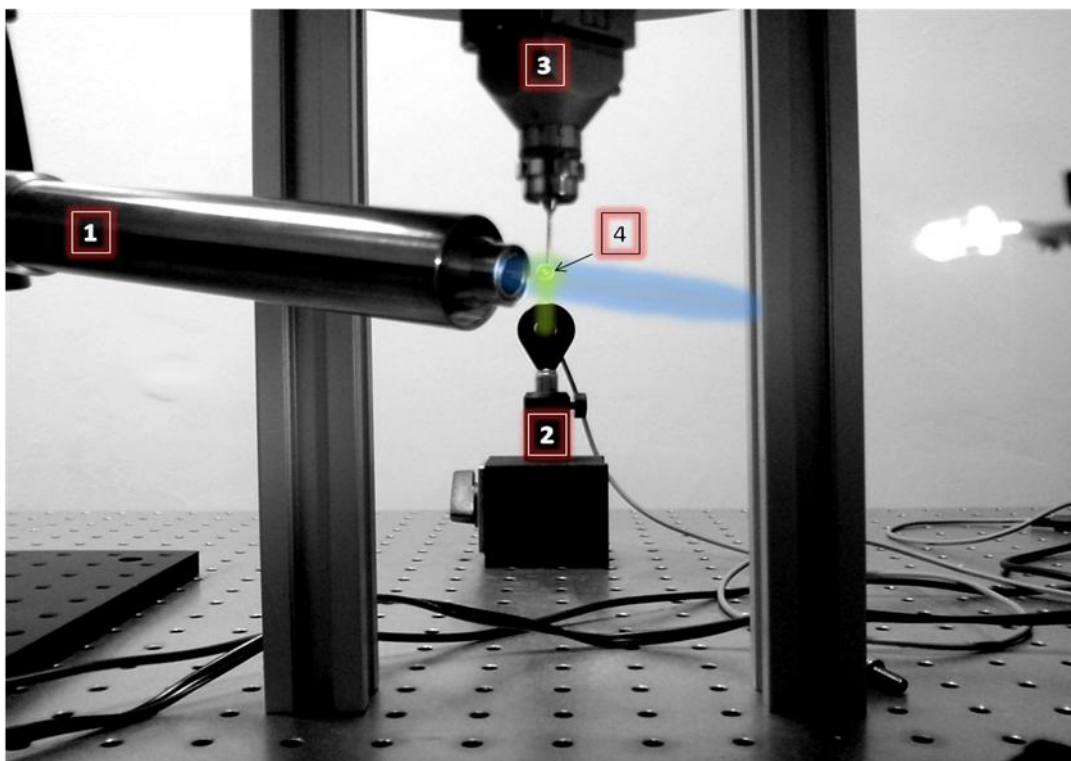


**Fig. 3** a) B12 crystals (image from the optical microscope), b) Crystal in the fiber loop

Crystals were selected under an optical microscope and harvested in nylon loops (figure 3b.). Highest quality crystals were chosen, harvested and afterwards stored in vials filled with liquid nitrogen. Fig. 3a shows an image of B12 crystals in a Petri dish under an optical microscope; the inset shows an enlarged view of the crystal. B12 crystallizes in an orthorhombic spacegroup with  $\alpha=\beta=\gamma=90^\circ$   $a\neq b\neq c$ . On the enlarged view one can note that the shape of the crystal is in agreement with this. Fig. 3b shows a picture of B12 crystal harvested in a fiber loop.

### 2.2.2 Laser irradiation experiment itself

Mounted B12 crystals were exposed to laser light for different times: 30, 60, 120, 180, 960 min. Lasers of two different wavelengths 405 nm and 532 nm were used. The irradiation set-up is shown in figure 5.

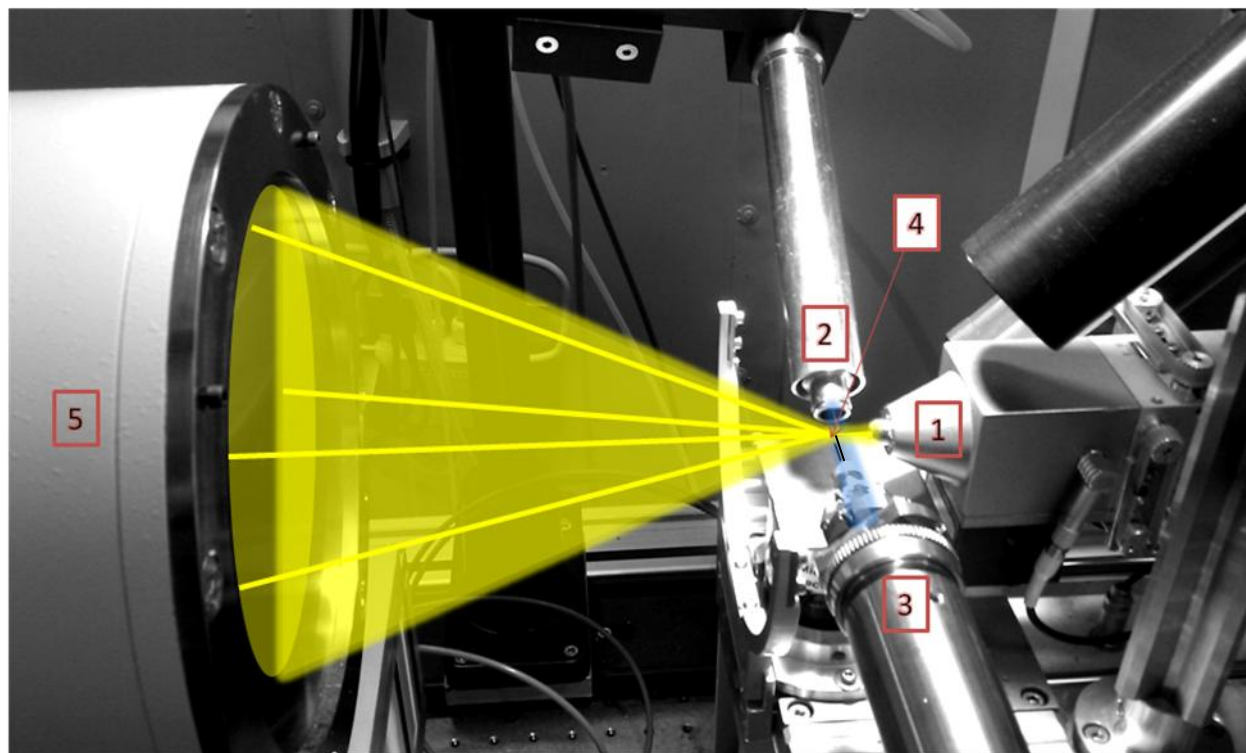


**Fig. 4** Picture of the irradiation set-up: 1) Cryo-flow of nitrogen,  $T=100$  K, 2) laser beam, 3) Sample-holder mounted on a Huber-circle, 4) Sample.

As it was noted before it's essential to perform all procedures in a cryo-flow. Therefore crystals were irradiated at a temperature of 100 K in order to prevent evaporation of the solvent from the crystals. To provide a uniform laser irradiation the crystal was rotated using a Huber-circle rotating at a speed of 2.5 °/sec during laser exposure. After the irradiation samples were put back in vials filled with liquid nitrogen and stored in storage Dewar.

## 2.3 X-ray Data collection

A scheme of the X-ray data-collection set-up (EMBL, beamline X13 at DORIS III at DESY) is shown in figure 5.



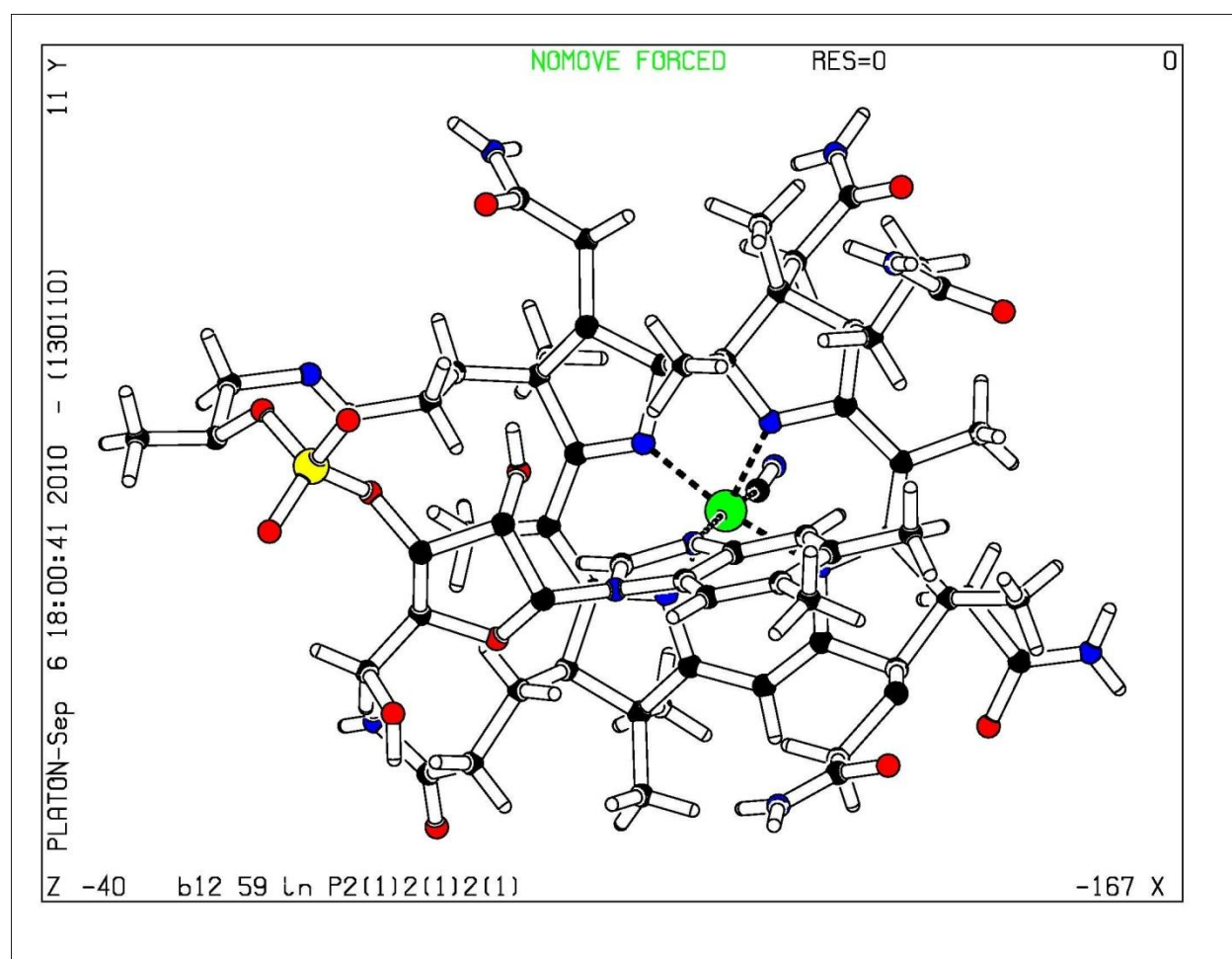
**Fig. 5** Data-collection set-up: 1) X-ray beam, 2) cold nitrogen flow, 3) sample-holder rotating the sample, 4) crystal mounted a nylon loop, 5) CCD detector.

Data collection parameters: 360 diffraction images were collected from every crystal. The crystal rotation increment per image was chosen to 1 degree. The radiation dose per image was set to 100 kHz; the wavelength was 0.8123 Å. Samples were cooled to 100 K during data collection.



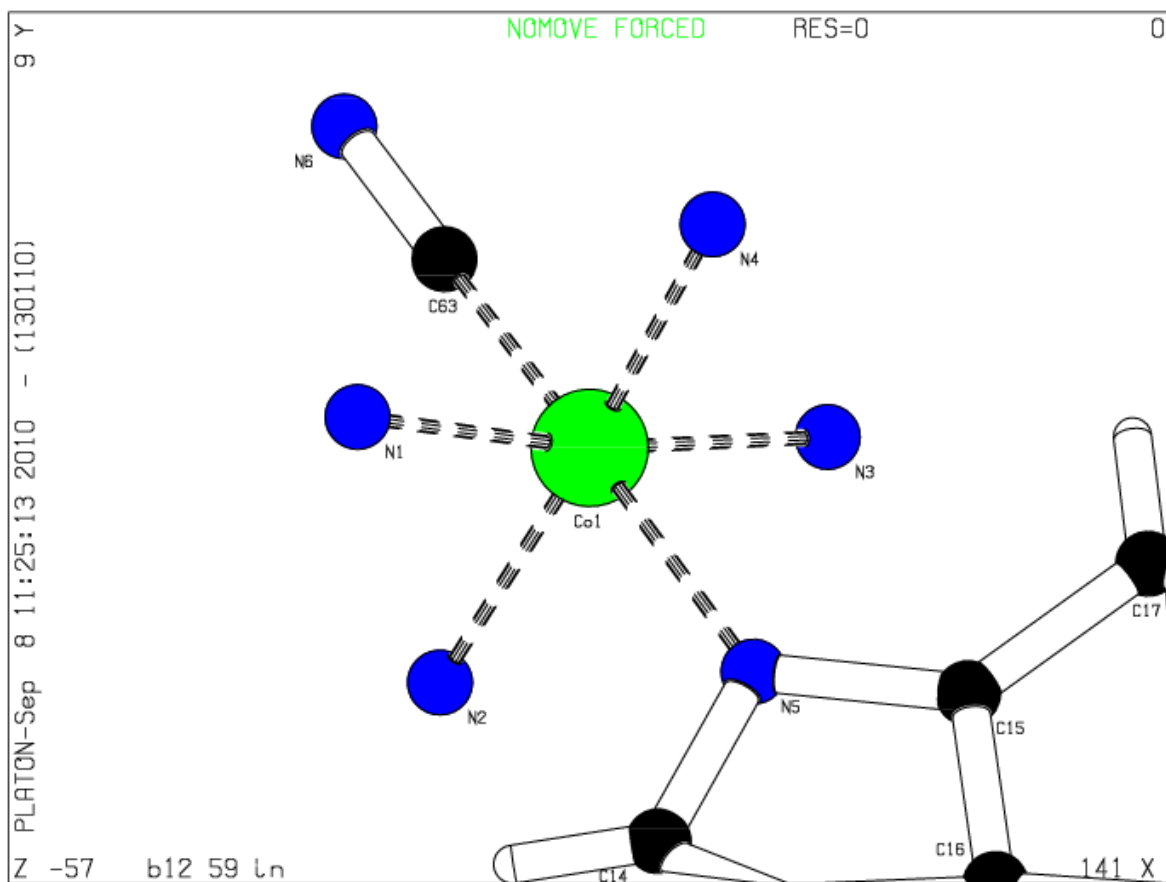
### 3 Results and discussion part

Diffraction images were initially processed with the XDS program package. Our B12 crystals crystallize in an orthorhombic primitive lattice in space group  $P\ 2(1)\ 2(1)\ 2(1)$  with lattice parameters of  $a=15.8$ ,  $b=22.6$ ,  $c=25.5$ ,  $\alpha=\beta=\gamma=90$ . The measured intensities of the different reflections were written into a called \*.hkl file. Structure refinement was done using the SHELX program. A general view of the B12 structure using PLATON is shown in figure 6a. The cobalt center with its six coordinating atoms and there labeling scheme is shown figure 6b. Bond lengths were extracted from a \*.cif file created by SHELXL.



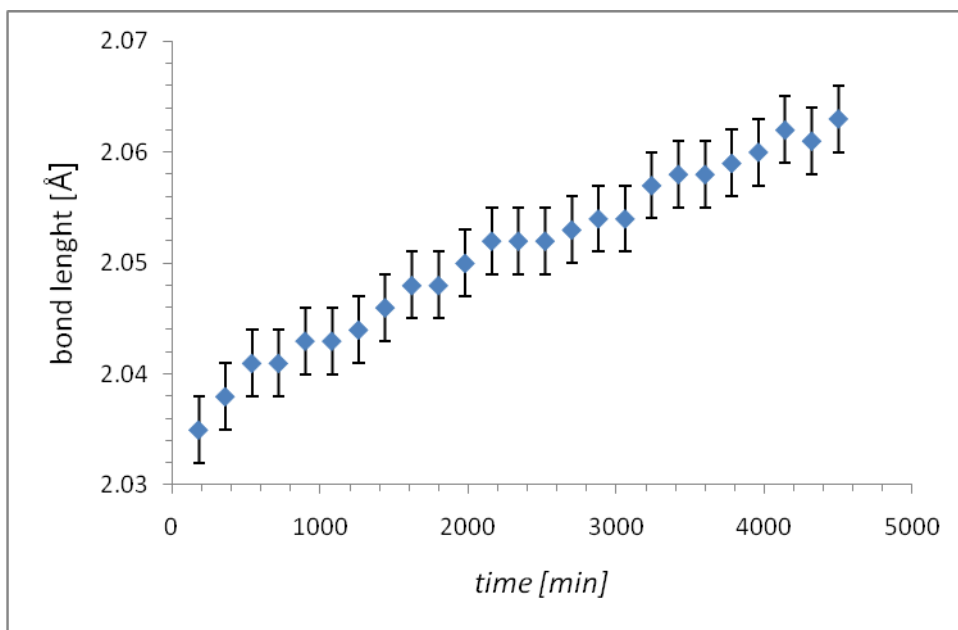
**Fig. 6a)** B12 structure visualized with PLATON after structure refinement

● -Co atom, ● -O atoms, ● -N atoms, ● -P atoms, ● -C atoms



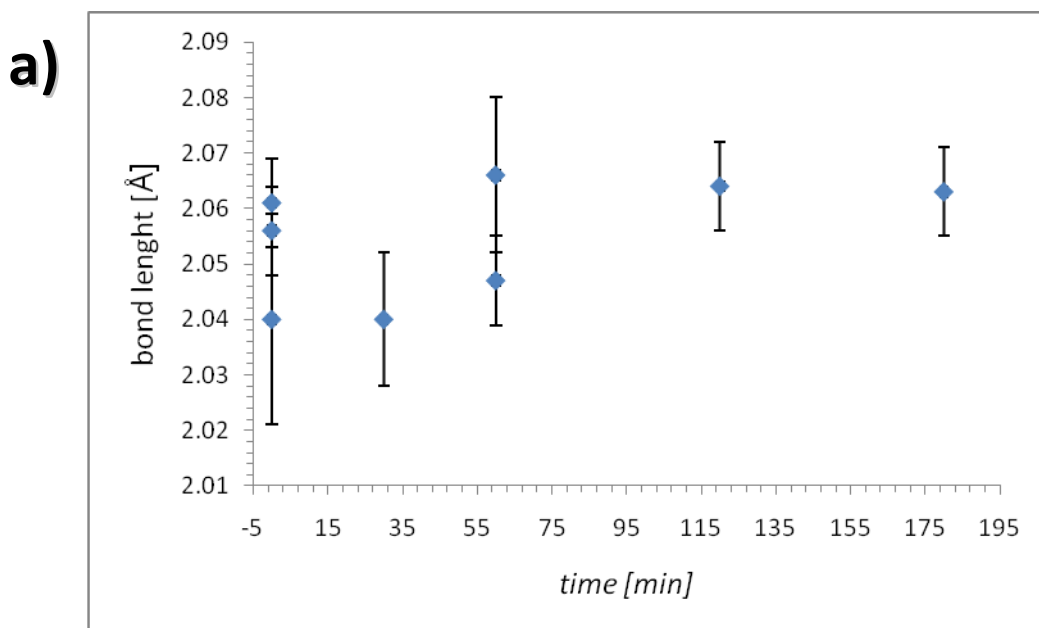
**Fig. 6b)** Cobalt center with its six coordinating atoms

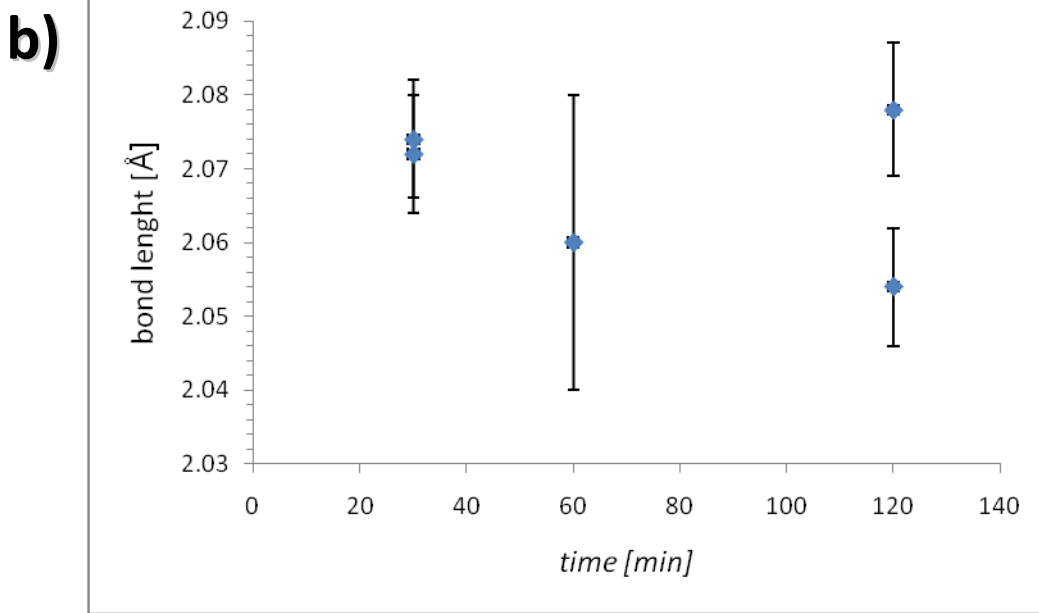
From previous experiments it is known that the Co-N5 bond is sensitive to X-ray photo reduction of vitamin B12 (fig. 6b). As shown in figure 7 the bond length between Co and N5 increases with X-ray irradiation time. This is probably a result of X-ray induced photo reduction of  $\text{Co}^{3+}$  to  $\text{Co}^{2+}$ .



**Fig. 7** Bond-length change of the Co-N5 bond in cobalamin with increasing X-ray irradiation time.

It was the goal of this work to see whether laser beam irradiation at wavelengths of 532 nm and 405 nm result in the same bond lengths changes. Our results are shown in figures 8a and 8b and in table 1.





**Fig. 8** Co-N5 bond-lengths of different B12 as function of laser irradiation time with wavelengths of : a) 405 nm ,and , b)532 nm. Each blue data point represents data from one crystal.

**Table 1:** Summary of X-ray diffraction data of all measured samples

sample	R-value	wavelength [nm]	time [min]	Co-N length [Å]	error of Co-N length [Å]
B12_11	0,23	532	0	2,061	0,008
B12_22	0,27		0	2,056	0,008
B12_59	0,33		0	2,04	0,019
B12_4	0,28		30	2,04	0,012
B12_5	0,09		60	2,047	0,008
B12_7	0,25		60	2,066	0,014
B12_36	0,21		120	2,064	0,008
B12_66	0,25		180	2,063	0,008
B12_04	0,21	405	30	2,072	0,008
B12_05	0,16		30	2,074	0,008
B12_16	0,32		60	2,06	0,02
B12_10	0,18		120	2,078	0,009
B12_26	0,15		120	2,054	0,008
B12_32	0,18		940	2,07	0,009

No significant bond lengths changes as function of irradiation time could be observed. The quality of the X-ray data collected and resulting X-ray structures are of relatively poor quality leading to high uncertainties in the bond lengths obtained. Better data quality might be obtained by improving the irradiation procedure and the whole process of cryo-handling. The mounting and irradiation procedure was done for the first time in the lab. It's quite challenging and requires a lot of precision. Another optimization procedure could be direct illumination of the crystal on the beamline. It's worth noting that for the samples with the same irradiation time observed bond-length differs from sample to sample, probably a result of the relatively poor data quality. Another reason for the fact that no significant bond length changes were observed could be the two wavelength (405 nm and 532 nm) used in our experiment. According to Ref. [6] illumination at these wavelengths does not lead to any changes in the absorption spectrum of cobalamin and hence probably neither does it lead to photo reduction.

Unfortunately, we were unsuccessful in getting diffraction images for B12 crystals grown in the dark and with  $\text{H}_2\text{O}_2$  so we cannot say anything about significant changes in these samples.

## 4 Conclusions

- The best ratio for the growing high-quality crystals for X-Ray crystallography is  $\text{H}_2\text{O}:\text{i-PrOH} = 1:[1.5;1.75]$
- No significant bond length changes and hence no photo reduction caused by laser light irradiation at 405 nm and 532 nm could be observed for exposure times of up to 16 h. Therefore future investigations should be carried out at shorter wavelengths around 350 nm [6].

## 5 References

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