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# **Accelerator analyses of tributyltin chloride associated with a tributyltin resistant marine microorganism**

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Organotin compounds such as tributyltin oxide, tributyltin chloride (TBTCI), and triphenyltin chloride are potent antifouling agents. Many self-polishing antifouling paints containing these compounds were developed and used worldwide in 1970s to prevent fouling on ships' bottoms. In the same era, the toxicity of such organotin compounds was reported in France (Champ & Lowenstein, 1987). Severe shell thickening in Pacific oysters and abnormally progressed male characteristics, *e.g.*, formation of a penis and a sperm duct in females of common dogwhelks (imposex) were observed in Arcachon Bay, France. Horiguchi *et al.* (1995) reported that 30 of the 38 species of Japanese gastropods were affected by imposex, and it could be induced in adult females of *Thais clavigera* exposed to seawater containing just 1 ppt ( $10^{-12}$  g/ml) of organotin compounds. As a result, the possibility that tributyltin compounds can act as endocrine disrupters has been recognized (Oberdorster *et al.*, 1998). Organotin compounds also have mutagenicity (Hamasaki *et al.*, 1993). Therefore, the International Maritime Organization has prohibited the usage of organotin compounds as antifouling agents after 2008.

Organotin compounds released from ships' bottoms are stable, and exist in marine environments for prolonged periods (Tao *et al.*, 1999), and this may cause an increase in frequency for the occurrence of tributyltin resistant marine microorganisms (Suzuki *et*

*al.*, 1992). A TBTCI resistant marine *Vibrio*, which can grow in the presence of 125  $\mu$ M of TBTCI, has been isolated from coastal seawater (Fukagawa *et al.*, 1992).

In order to clarify the degradation processes in TBTCI resistant marine microorganisms, development of the analytical instrument is necessary to detect cell associated TBTCI and the small amounts of the biodegradation products. Gas chromatography-mass spectrometry (GC-MS) is conventionally used to quantify organotin compounds and degradation products such as dibutyltin and monobutyltin chloride with the sensitivity of a few ppb (Tao *et al.*, 1999). Some pre-treatment procedures of the samples, which cause an increase in experimental error, are necessary to quantify organotin compounds, especially those associated with giant molecules or microorganisms.

Analyses using an accelerator (particle induced X-ray emission analysis (PIXE), particle induced  $\gamma$ -ray emission analysis (PIGE) and Rutherford backscattering spectroscopy (RBS)) can provide direct information on the composition of atomic elements, including isotopes. Moreover, there are few pre-treatment processes required when these methods are used for the measurement of biological samples. Therefore, in the present study, we have applied the accelerator analyses to the quantification of Sn, Cl and Na elements associated with a TBTCI resistant marine

microorganism that was newly isolated from sediment in a ship's ballast water tank.

An outline of the sample treatment procedure is shown in Fig. 1. The isolate was pre-incubated at 30°C in a medium containing 5 g Bactopeptone (Difco, Michigan, USA), 1 g yeast extract (Difco), 0.4 M NaCl, 10 mM KCl, 10 mM CaCl<sub>2</sub>•2H<sub>2</sub>O, 53 mM MgCl<sub>2</sub>, 28 mM Na<sub>2</sub>SO<sub>4</sub> and 30 µM TBTCI. Medium pH was adjusted to 7.8 using tetramethylammonium hydroxide. After 1 day incubation, 1 ml of cell suspension was added to 100 ml of a medium similar to that described above, except it contained a final concentration of 150 µM TBTCI.

An aliquot of cell suspension (1 ml) was taken after 1, 3, 5 and 7 days of incubation, and then centrifuged (10,000 rpm, 5 min) at 4°C. Washing was carried out three times with distilled water, and cells were re-suspended in 1 ml of distilled water. Samples thus obtained were stored at -20°C until accelerator analyses.

Subsequently, samples were dried in atmospheric environment as follows. Each sample was slowly dropped into a hollow of 2 mm diameter on a carbon base by pipetting, and then gently dried for 40 minutes. Some 70 repetitions of this process were required to finish loading the 1 ml sample, during which process the volume of the sample was drastically reduced to become hardly visible.

Total viable cell numbers per ml were analyzed in one sample following 1/10 serial dilution with 50 mM potassium-phosphate buffer, pH 7.5. The resulting cell suspension was spread onto agar plates containing nutrients and inorganic salts as described above, but no TBTCI. Colonies formed were counted after 2 days of incubation at 30°C.

Three methods of accelerator analyses were applied simultaneously. Areal densities of cell associated Sn and Cl were measured by means of PIXE. Sodium atoms which were added to the medium as NaCl and are essential for the growth of the isolate seem to be contained in the cells. Since the PIXE method has difficulties in detecting characteristic X-rays from atoms with relatively small atomic numbers such as Na, PIGE was performed to determine Na density in order to eliminate the background contribution of Cl originated in the NaCl. RBS was utilized to measure the near surface composition and thickness of the sample target.

The experimental setup is shown in Fig. 2. The samples were analyzed in the vacuum chamber connected to the M30 beam line of a 5SDH-2 pelletron accelerator under a pressure of  $1.0 \times 10^{-4}$  Pa. The sample target was exposed to a probe beam of 3.0 MeV protons at an incident angle of 0° with a beam current of 2 nA. Proton incidence of 3.0  $\mu$ C was necessary to obtain reasonable statistical accuracy of X-ray

yields from Sn and Cl. Occasionally, 4.5 MeV  $\text{He}^{++}$  with an incident charge of 20  $\mu\text{C}$  was used as the probe beam for RBS exclusively. A beam spot diameter (2 mm) was determined from color change of a dummy target of aluminized polyester. A lithium-drifted silicon (Si(Li)) detector for PIXE and a silicon surface barrier detector (SSBD) for RBS were positioned at  $\pm 130^\circ$  angles to the incident direction of the probe beam. A piece of aluminum foil with 18  $\mu\text{m}$  thickness was mounted in front of the Si(Li) detector to reduce low-energy background X-rays. A thallium-activated sodium iodide (NaI(Tl)) scintillation counter for PIGE was positioned at an angle of  $50^\circ$ . Solid angles subtended by the Si(Li) detector, SSBD and NaI(Tl) scintillation detector were  $5.4 \times 10^{-2}$ ,  $2.0 \times 10^{-3}$  and  $1.2 \times 10^{-1}$  sr, respectively.

In addition to the cells prepared as described above, a 1-mm-thick Sn sheet, crystallized NaCl, the carbon base itself and a sample prepared from the cell suspension at stationary phase of growth in the absence of TBTCI were also analyzed.

In the PIXE analysis, atomic species existing in the sample are identified by the energy of X-rays, while atomic density of a species of interest is deduced from the number of  $K_\alpha$  X-rays detected by the Si(Li) detector,  $Y_X$ , which is expressed as

$$Y_X = Q \int_0^t n(x) \frac{\sigma_i(E)}{4\pi} \Delta\Omega \times [\omega_K k_\alpha] \times \left[ \exp\left(-\frac{\mu x}{\cos\theta}\right) \cdot T \right] \varepsilon dx, \quad (1)$$

$$= Q \bar{n} \frac{\bar{\sigma}_i}{4\pi} \Delta\Omega \omega_K k_\alpha T \varepsilon \cdot t_{\text{eff}}, \quad \left( t_{\text{eff}} \equiv \frac{\cos\theta}{\mu} \left[ 1 - \exp\left(-\frac{\mu t}{\cos\theta}\right) \right] \right), \quad (1')$$

where  $Q$ ,  $n(x)$ ,  $\sigma_i(E)/4\pi$  and  $\Delta\Omega$  are the number of incident particles, depth ( $x$ ) dependent atomic density of a species of interest, K-shell ionization cross section by the projectile with energy  $E$  and the solid angle of the detector, respectively. The cross section  $\sigma_i(E)$  is approximated with a polynomial of  $E$  (Mayer & Rimini, 1977). The second term of the integrand denotes X-ray emission probability, where  $\omega_K$  and  $k_\alpha$  are fluorescence yield from the K-shell and fractional transition probability of  $K_\alpha$ -line to all K-lines, respectively (Mayer & Rimini, 1977). The third term expresses X-ray attenuation due to absorption in the sample itself, windows and filters located between the sample surface and the detector, where  $\mu$ ,  $\theta$  and  $T$  are the X-ray absorption coefficient of the sample itself, the detection angle and the X-ray transmittance for the absorbers, respectively. Finally,  $\varepsilon$  is the X-ray detection efficiency of the detector. The integration is done over the sample with thickness of  $t$ . Eq. (1') is successfully adopted as an approximation for cases in which the mean free path  $\mu^{-1}$  of the emitted radiation is comparable to or smaller than the thickness  $t$ , where  $\bar{n}$ ,  $\bar{\sigma}_i$  and  $t_{\text{eff}}$  denote

the average density, the average cross section and effective thickness of the sample, respectively.

The transmittance  $T$  is calculated with the following formula

$$T = \prod_j \exp(-\mu_j \lambda_j) = \prod_j \exp\left(-\sum_i \sigma_{\text{ph},i} n_{i,j} \lambda_j\right), \quad (2)$$

where  $\sigma_{\text{ph},i}$  is the total X-ray interaction cross section of an  $i$ th element with an atomic density  $n_{i,j}$  in the  $j$ th absorber having the absorption coefficient  $\mu_i$  and the thickness  $\lambda_j$ .

Energy dependence of  $\sigma_{\text{ph}}$  for each element is expressed as power series of logarithmic energy (MacMaster *et al.*, 1969).

In the present experimental setup shown in Fig. 2, the X-ray produced in the sample target reaches the detector through a Kapton foil, an air layer and a beryllium window. The values of  $T$  are then  $5.6 \times 10^{-4}$  and 0.99 for Cl- $K_\alpha$  (2.6 keV) and Sn- $K_\alpha$  (25.3 keV) X-rays. The value of  $\varepsilon$  is unity for the present Si(Li) detector.

The secondary effect, fluorescence induced by primary X-rays, which increases number of X-rays having lower energies, is neglected in Eq. (1). This could result in overestimation of density of less abundant elements having lower atomic numbers. In the present case, however, this introduces negligible error to the result, since in the

present samples the lighter element of interest has a concentration of the same order of magnitude as the heavier one.

For PIGE, the case is almost the same as above, except that the cross section  $\sigma_i(E)$  is replaced by the nuclear reaction cross section and other terms in Eq. (1) regarding emission and absorption probabilities are replaced by unity. In general, the reaction rate becomes negligible below a threshold energy of the projectile. The approximation (1') is used here for cases where the density and the cross section do not vary so much to the depth where the projectile is decelerated down to the threshold energy.

In the case of RBS, energy of the scattered particles depends not only on the mass of the target atom but also on the depth at which the scattering reaction took place, which enables profiling of atomic density. Deduction of the density profile of a specified atom is performed with use of a versatile software "TranScat-RErNPro". The first half of the program provides a table of yield-energy relation of scattered particles, recoil particles and reaction products as a function of depth in a layered sample with a specified composition, while the latter half converts the energy spectra to density profiles. The calculation is based on Eq. (1) with the cross section replaced by the double differential cross section  $d\sigma(E)/d\Omega$  for scattering (RBS), recoil (ERDA) and

nuclear reaction (NRA/PIGE), and with emission and absorption probabilities replaced by unity. Basically the scattering cross section  $d\sigma(E)/d\Omega$  is expressed by the Rutherford formula, but an enhancement factor should be taken into account in some regions of the energy at which resonance nuclear reaction occurs (Mayer & Rimini, 1977).

Changes in the total viable cell numbers of the isolate throughout the incubation period both in the presence and absence of TBTCI are shown in Fig. 3. The maximum value reached at the early stationary phase of growth was repressed to about one-tenth in the presence of 150  $\mu$ M TBTCI in comparison with that in the absence of TBTCI.

A typical He-RBS spectrum obtained from the sample taken after 3 days of incubation in 150  $\mu$ M TBTCI is shown in Fig. 4. In the spectrum the elements N, O, Na, Cl and Sn can be identified in the energy region higher than 1.5 MeV. These elements may originate in the cells or the medium. The bottom and the top of the arrow are located at the energies of He ions scattered by the specified element existing at the surface of the sample and at a depth of 1  $\mu$ m in the sample, respectively.

The yields were corrected for overlapping by those from the adjacent element to evaluate the atomic density of each element. Here we have assumed constant density

distributions beyond the depth corresponding to the overlapping energies. Assuming a specific weight of the sample as 1 g/cm<sup>3</sup>, and neglecting all other elements, the densities of C, N, O, Na, Cl and Sn in the near surface region thus calculated were 400, 49, 16, 4.3, 2.5 and 0.32 ×10<sup>20</sup>/cm<sup>3</sup>, respectively. This composition was used for determination of sample thickness and X-ray intensity correction for self-absorption. In view of the above process of data manipulation, an uncertainty of a factor of 2 in the density values for each element heavier than C should be assumed, while a factor of 1.3 is assumed for C.

Gamma-ray energies of <sup>23</sup>Na(p,  $\gamma$ p<sub>1</sub>)<sup>23</sup>Na, <sup>23</sup>Na(p,  $\gamma$  $\alpha$ <sub>1</sub>)<sup>20</sup>Ne, <sup>35</sup>Cl(p,  $\gamma$ p<sub>1</sub>)<sup>35</sup>Cl and <sup>37</sup>Cl(p,  $\gamma$ <sub>3-1</sub>)<sup>38</sup>Ar reactions are 0.44, 1.63, 1.22 and 1.64 MeV, respectively (Tesmer & Nastasi, 1995). A typical PIGE spectrum for the sample at 5 days incubation is shown in Fig. 5. We can identify the  $\gamma$ -rays from these reactions except the <sup>35</sup>Cl(p,  $\gamma$ p<sub>1</sub>)<sup>35</sup>Cl reaction, the cross section of which is greater than that of the <sup>37</sup>Cl(p,  $\gamma$ <sub>3-1</sub>)<sup>38</sup>Ar reaction by two orders of magnitude. Therefore, we infer that the Cl density in the sample is rather small and the peak near 1.6 MeV is dominated by the  $\gamma$ -rays from the <sup>23</sup>Na(p,  $\gamma$  $\alpha$ <sub>1</sub>)<sup>20</sup>Ne reaction.

The  $\gamma$ -ray yield from the <sup>23</sup>Na(p,  $\gamma$ p<sub>1</sub>)<sup>23</sup>Na reaction is used to calculate the average density of Na in each sample by Eq. (1'). Unfortunately the energy dependent cross

section of this reaction cannot be found in literatures, to author's knowledge. From a yield value for a thick target of Na (Mayer & Rinini, 1977), we have deduced a cross section of 4.9 mb averaged over an energy region between 3.1 and 2.4 MeV. The Na densities thus calculated lie between  $10^{20}$  and  $10^{21}/\text{cm}^3$  as a function of incubation period (see also Fig. 7). It should be noted that the  $\gamma$ -ray yield from the reference sample of crystalline NaCl together with the above value of the cross section gives a Na density in agreement with the nominal density of  $2.2 \times 10^{22}/\text{cm}^3$  with an uncertainty of a factor of 1.5.

A typical PIXE spectrum, which was taken from the sample after 5 days incubation, is shown in Fig. 6.  $K_\alpha$  and  $K_\beta$  lines of Sn with energies of 25.3 and 28.6 keV, respectively, have been measured with a yield ratio of  $Y(K_\alpha)/Y(K_\beta) = 9 \pm 0.9$ , which is consistent with the published value (Mayer & Rinini, 1977). The 2.6-keV peak of Cl- $K_\alpha$  line is also identified on the background continuum. In addition, the most intense peak around 3.6 keV is formed by Sn- $L_\alpha$  with 3.443 keV and Ca- $K_\alpha$  with 3.690 keV. The other eight peaks reveal the existence of six elements. Aluminum used as a part of the target holder seems to be a contaminant, while P, Fe, Cu, Zn and Br seem to be the cell originated elements.

Since the TBTCl itself is rather volatile, temperature rise of the sample due to ion

irradiation could cause loss of TBTCI molecules. However, variation of the peak yields of Sn per unit fluence of incident protons was less than 3%. Sample degradation or Sn compound release during ion irradiation is therefore negligible, which indicates that the Sn compound is rather tightly associated with the cells. This necessitates no correction of the raw data for the ion-induced degradation of the sample.

The energy of the Sn-K $_{\alpha}$  line is high enough for the photons to penetrate with no attenuation through the cell sample having the atomic composition described previously, even with a thickness of the order of mm. Essentially no correction of intensity for self-absorption is therefore necessary. Using Eq. (1'), the areal density of Sn,  $n_{\text{Sn}}t$ , in the sample after 3 days incubation is calculated to be  $1.4 \times 10^{16} \text{ cm}^{-2}$ . Dividing this value by the Sn density obtained from the RBS spectrum, we determine the sample thickness  $t$  to be 4.2  $\mu\text{m}$ .

The Cl-K $_{\alpha}$  intensity should be subjected to the self-absorption correction of  $t_{\text{eff}}/t = 1.1$  for the cell samples of this thickness. Assuming that all samples have the same thickness, the densities of Cl,  $n_{\text{Cl}}$ , are calculated with use of Eq. (1'), and shown as a function of incubation period in Fig. 7 together with those of Sn and Na. The lines connecting the data points are a guide for the eye only. Also shown are the Na and the Cl densities in the cell sample incubated in the absence of TBTCI, and the values for the

3 days incubation sample derived from the RBS spectrum. The typical uncertainty associated with the data is a factor of 3 including a factor of 2 above mentioned previously.

The density of Sn increases monotonically with increase in the incubation period. When this is compared with Fig. 3, it can be seen that even dead cells adsorb Sn compounds. The Na density has a dependence similar to the Sn density except for the sample at 3 days incubation.

On the other hand, the Cl density has no apparent dependence on the incubation period. The Cl density in the samples of 3 and 7 days incubation is much smaller than the Na density. It should be noted that the ratio of the Cl density to that of Na is smallest in the sample of cells incubated in the absence of TBTCI. There may be a possibility that Cl atoms could be liberated out of the system in the form of a gas as a result of Na intake by the microorganism as a biological activity. The relative abundance of the Cl atoms in the cell samples incubated in the presence of TBTCI should be attributed to the cell associated Sn compounds. Except for the 3 days incubation sample, the differences exceed the density of Sn atoms, which suggests that some of the butyl bases of the TBTCI have been replaced by Cl.

The total number of Sn atoms associated with the cells is calculated to be  $4 \times 10^6$

in the sample of the 3 days incubation, for example, by using the viable cell number of  $1.4 \times 10^8$  cfu and the number of Sn atoms,  $4 \times 10^{14}$ , located on the beam spot area of  $0.03 \text{ cm}^2$ . We assume here that the TBTCI resistant microorganism does adsorb, not absorb, Sn compounds onto the surface of the cells. Since the surface area of the cells is about  $2 \times 10^{-8} \text{ cm}^2$ , an area occupied by a single Sn compound is calculated to be  $7 \times 10^{-15} \text{ cm}^2$ , which corresponds to an equivalent diameter of 0.5 nm. If we assume that all Sn atoms are adsorbed in the form of TBTCI, it seems that there is little area left for the cell surface to transport nutrients. It would therefore be possible that the TBTCI molecules on the cells might have been transformed into other Sn compounds with a smaller size. This is consistent with the speculation in the last paragraph that some of the butyl bases have been replaced by Cl. In spite of the large uncertainty of a factor of 3, the general tendency of these data supports a hypothesis that the present microorganism can degrade TBTCI molecules.

A marine *Pseudomonas chlororaphis* (Yamaoka *et al.*, 2002) can degrade dibutyltins to monobutyltins by pyoverdins, which have a molecular mass of 1 - 1.5 kDa and constitute a chromophore (Budzikiewicz, 1997). The compound, however, can not degrade TBT and monobutyltin compounds. The present TBT resistant isolate might have another mechanism to cope with the toxicity of organotin compounds.

Finally, although sensitivity is a matter of little concern for the samples in the present work, it is worth while discussing this matter for advanced application in future. The number of Sn atoms detected for the sample after 3 days incubation was  $4 \times 10^{14}$ , which corresponds to  $2 \times 10^{-7}$  g ( $7 \times 10^{-10}$  mol) of TBTCl associated with the cells in 1 ml of the medium. These values correspond to a concentration of 200 ng-TBTCl/ml (700 nM), or 4 ppb in terms of atomic fraction of Sn.

These are based on the  $K_{\alpha}$ -line yield of 9900 counts/1500 s. Applying the three-sigma criterion, in which the detection limit is defined as the level of the background yield (600 counts/1500 s in the present case within this energy region) added by three times of standard deviation, we find the sensitivity to be 6% of the above values; 10 ng-TBTCl/ml (40 nM), or 200 ppt in terms of atomic fraction of Sn, for a modest analyzing time of 1500 s. The sensitivity could be improved substantially by reducing the background X-ray counting and increasing the detector solid angle.

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## Figure Captions

Fig. 1. Scheme of the experimental procedure.

Fig. 2. Scheme of target setup for accelerator analyses.

Fig. 3. Changes in viable cell number during incubation in the presence of 150  $\mu\text{M}$  TBTCI (closed circles) and in the absence of TBTCI (open circles). Growth experiments were carried out twice, and the averaged data are shown. The relative differences between the data were less than a factor of 2.5 throughout the experiments.

Fig. 4. Typical RBS spectrum obtained from the sample made after 3 days incubation in 150  $\mu\text{M}$  TBTCI, which shows existence of species indicated. The bottom and the top of each arrow shows the energy of  $^4\text{He}$  scattered by each atom located on the surface of the sample and at a depth of 1  $\mu\text{m}$ , respectively.

Fig. 5. Typical PIGE spectrum obtained from the sample made after 5 days incubation. Two peaks of gamma-rays originating in proton reaction with  $^{23}\text{Na}$  are identified, while the other two peaks are from natural radioisotopes.

Fig. 6. Typical PIXE spectrum obtained from the sample made after 5 days incubation. Other elements are identified besides Sn and Cl.

Fig. 7. Atomic density of Sn, Cl and Na as a function of incubation period. Those of

Sn (open circles) and Cl (open triangles) were deduced from the PIXE data, while those of Na (open squares) were from PIGE data. The data are shown as the averaged values between those from 3 samples which scatter by about a factor of 1.2, 3 and 1.3 for Sn, Cl and Na signals, respectively. For a sample of 3 days incubation, those from  $^4\text{He}$ -RBS are also shown; a closed circle for Sn, a closed triangle for Cl, and a closed square for Na. The double triangle and the double square at 1 day are those of Cl and Na, respectively, in the sample incubated in the absence of TBTCl.

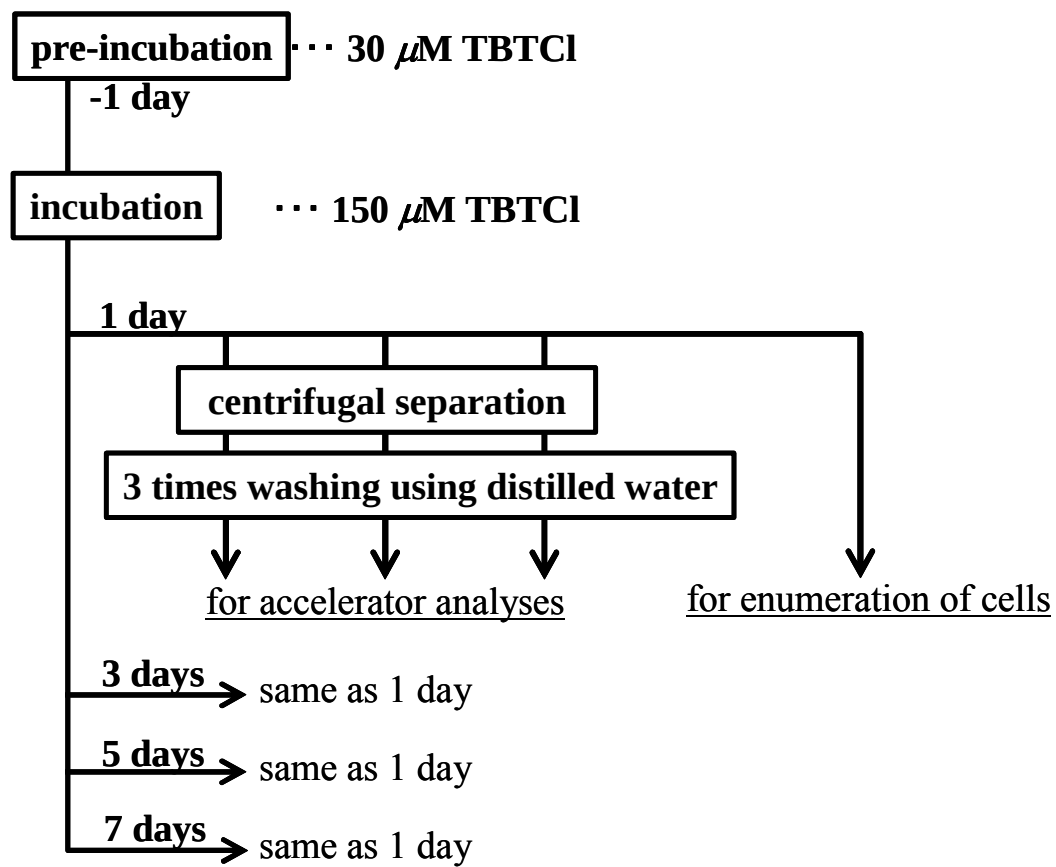
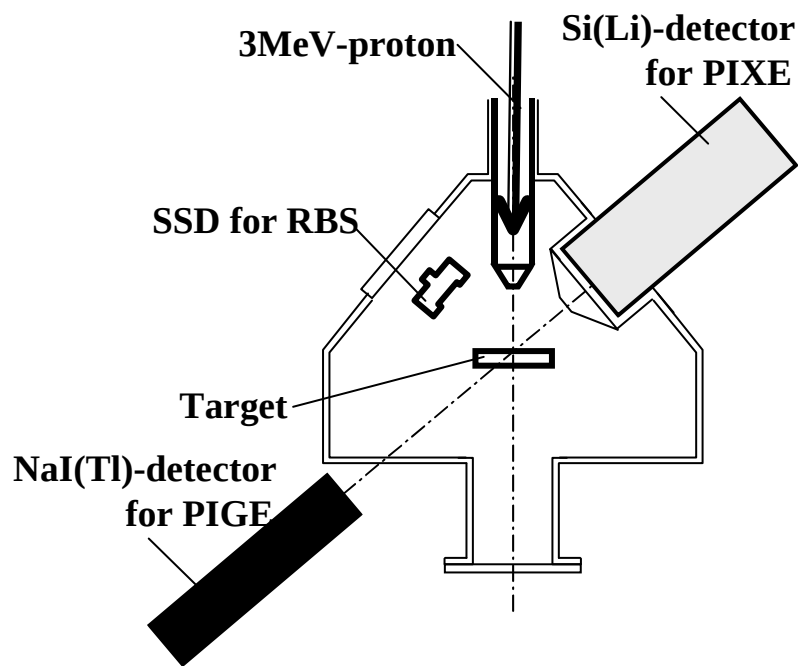


Fig. 1



enlarged view near the window for PIXE

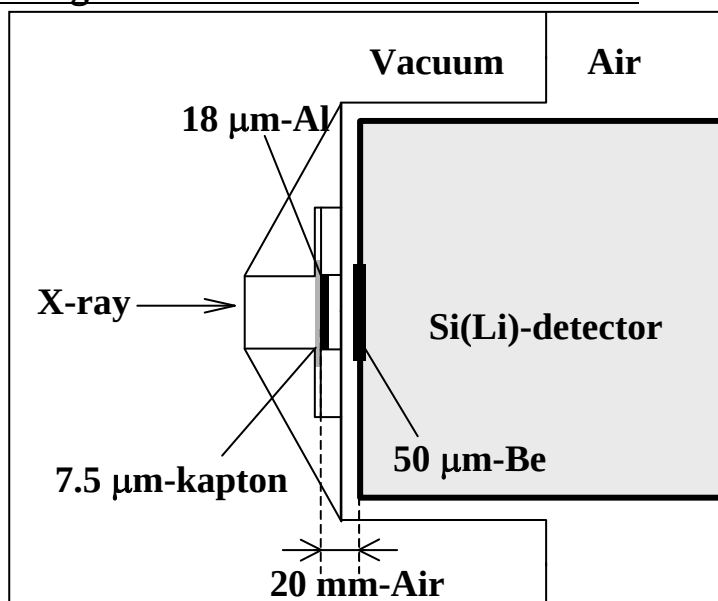


Fig. 2

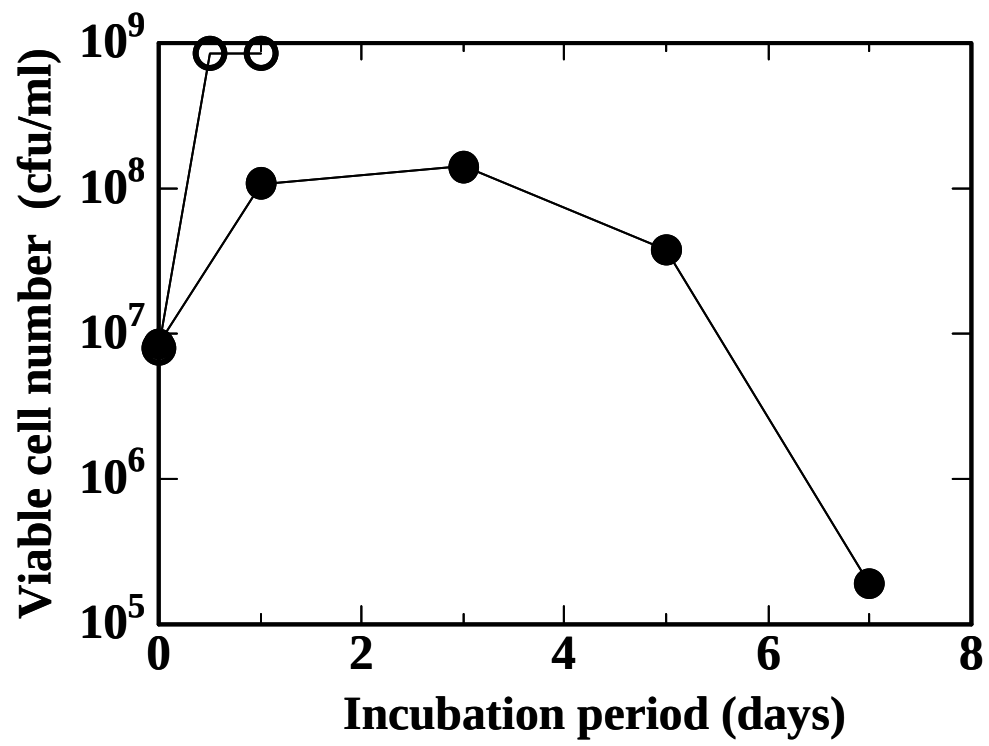


Fig. 3

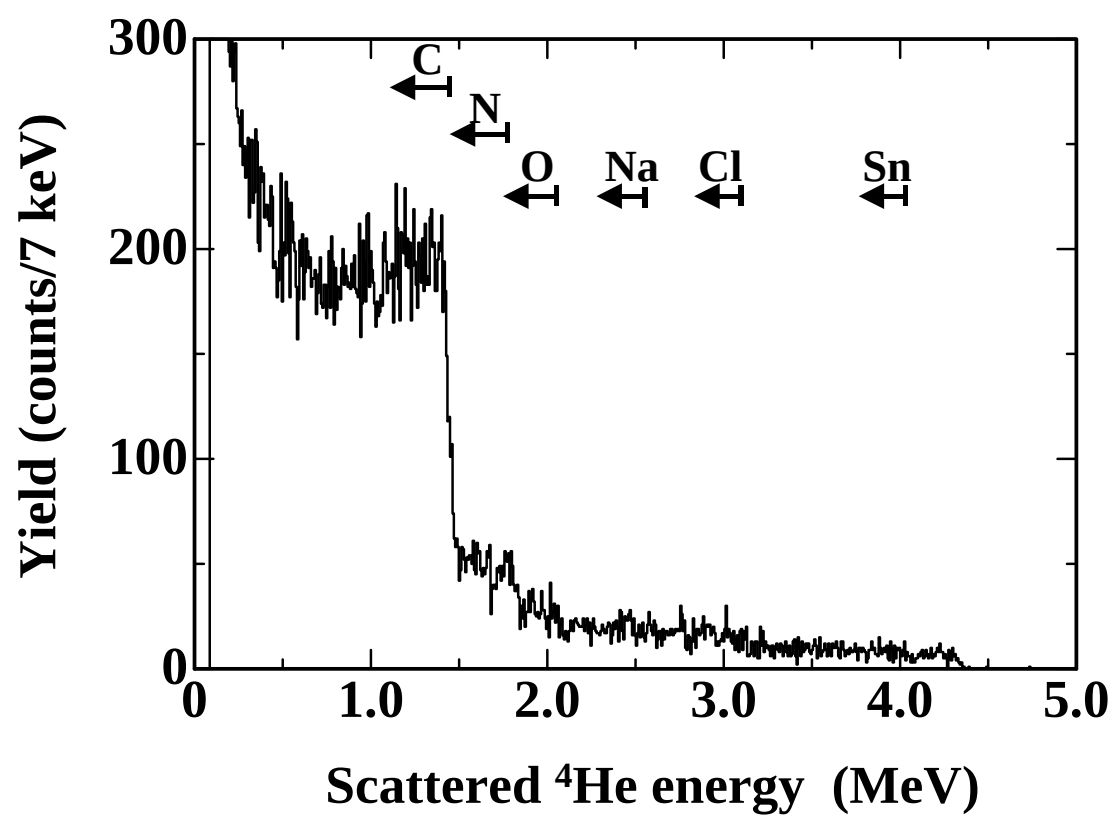


Fig. 4

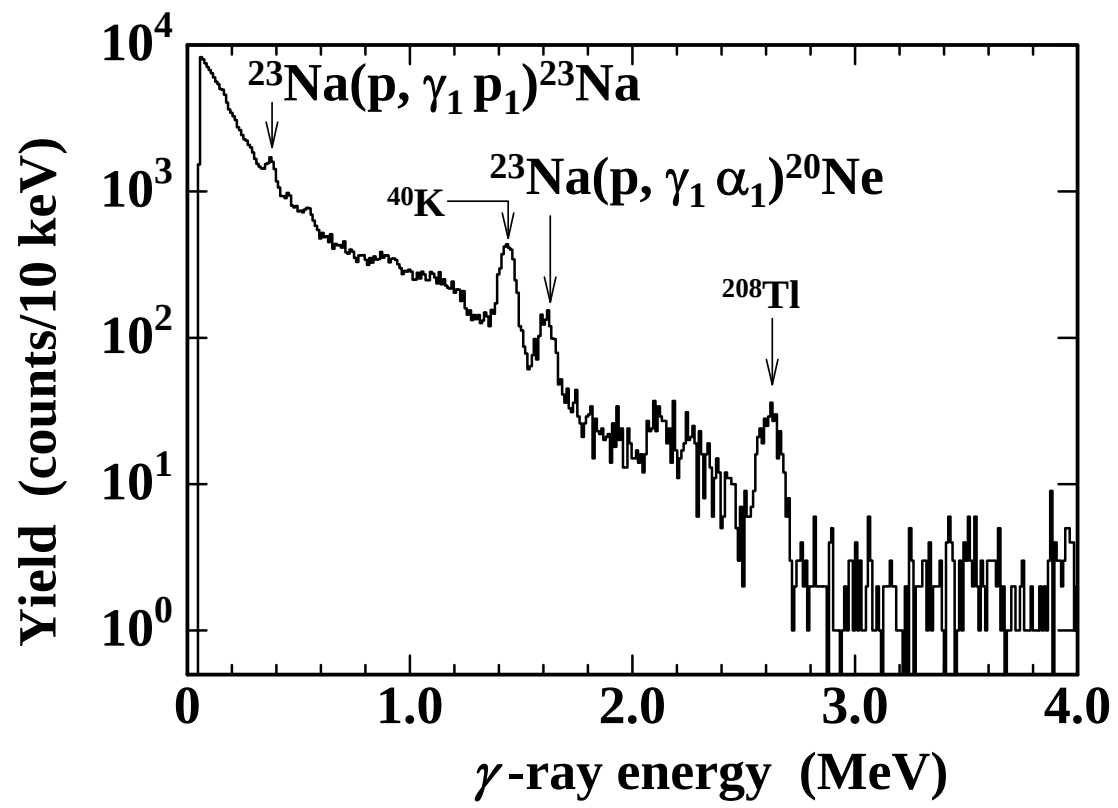


Fig. 5

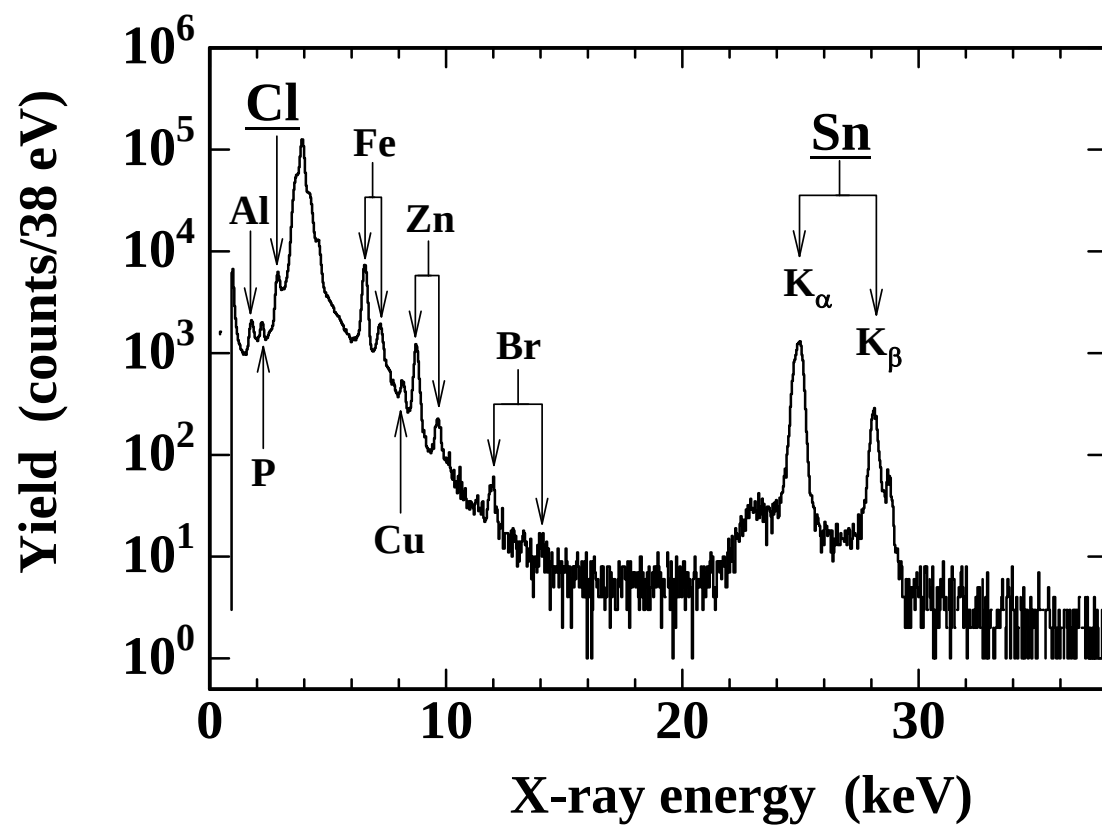


Fig. 6

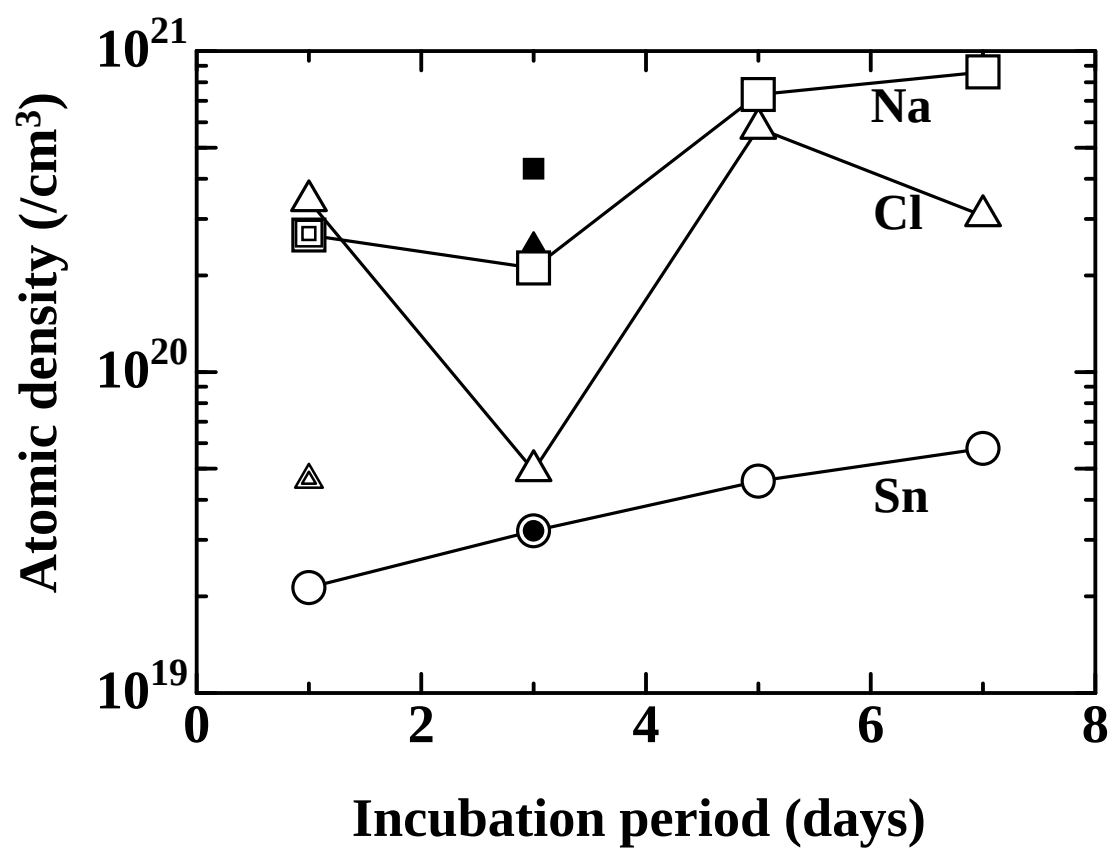


Fig. 7