# Chapter 14

# The Rapid Detection of Microbiological Activity using E-tATP

## 14.1 Introduction

E-tATP stands for enhanced total adenosine triphosphate which is the prime energy storage mechanism in all biological systems as well microorganisms. Energy is stored in the ATP rather akin to the storage of electricity in a battery. When a cell is active then it will use some of this stored energy but when it has grown then it will store surplus energy within ATP. Often in microorganisms cells will continue to save as energy as ATP to support even six or seven generations of daughter cells (i.e. from one cell with stored ATP to support n as many as 64 or 128 of these cells. That means (on the good side) that it is possible to detect the amount of stored energy in the cells present in the sample. On the bad side the detection is of the levels of energy stored in that form at that moment in time! Classical ATP testing can be performed at a number of levels from presence / absence through to quantification of either the total ATP or parts thereof. Parts that are sometimes measured are the dissolved fraction, factions that are bacterial in origin or particulate. The technique developed and known as the rapid biochemical oxygen demand represents an improvement in precision over the existing techniques. Precision is improved by triggering the production of ATP by the cells in the sample through enhanced culture. Enhancement as a technique involves triggering ATP production (x3 to x10 depending on population size and initial activity levels). This triggered production is achieved by placing 15mL of the sample in an ENH- Bart tester and then rotating it three times in one minute at room temperature. Although this time is short in our terms it is long enough for the bacteria to respond to the protein and phosphate rich culture medium by maximally generating stored energy as total ATP. Extending the rotation period to up to fifteen minutes does not generate the precision observed routinely after one minute. The standard protocol for the measurement of E-tATP therefore involves the ENH- tester being rotated at 3rpm for one minute.

This technology to determine the E-tATP represents a novel approach to the effective determination of the potential energy levels within the sample and allows the determination of whether there is enough bacterial activity to warrant further investigations using the Bart testers.

E-tATP activity can also be used to rapidly determine the biochemical oxygen demand in effluents from wastewater treatment plants. This testing is essential to assure that the discharge has a low and acceptable environmental impact and would supplement the use the various five day biochemical oxygen demand (BOD) tests that are commonly regulated by law. There are two stages in the rapid testing and this protocol relates to the rapid determination of oxygen demand using total adenosine triphosphate (tATP) in less than fifteen minutes. To improve the accuracy the sample is first subjected to the enhanced cultural triggering of ATP activity for one minute in the ENH – BART tester. This stage calls for the tester to be inverted every ten seconds and radically increases the production of total ATP as enhanced total ATP (or E-tATP). Confirmatory testing, if required uses the HAB- tester and the visual Bart reader (VBR) system which is described in Chapter 15. The next section (14.2) deals with the determination of E-tATP primarily for wastewater treatment final effluents but is equally applicable to the determination of E-tATP in samples that could be subjected to Bart testing.

**14.2 E-tATP determination**

E-tATP determination in a water or wastewater sample represents an improvement over the previous ATP testing methods by virtue of enhancing the bacterial activity recordable from the sample in a more precise manner. Presently testing for ATP activity is based upon the presence / absence (primarily qualitative) or the instant determination of the ATP or some fraction thereof (primarily quantitative). To date ATP testing has been designed to give a very rapid determination of the amount of ATP at that moment in time when the sample is taken and quickly tested.

Variability (precision) becomes a significant issue since there are natural variations in the metabolic states of the individual cells present in the sample (including those that are inactive to those that are very active). Improving precision of the ATP testing has been achieved by triggering ATP activity within the cells in the sample by the specified enhanced cultural strategy using the ENH- tester. This enhancement creates a very oxidative, turbulent and nutrient rich conditions for one minute to stimulate ATP production. Typically during that minute the ATP levels increase by x3 to x10 depending on the cell loading and potential for activity in the sample.

This one minute cultural enhancement method triggers the cells to maximise their energy storage potential in anticipation of "favourable growth conditions". To do this, the technology uses the enhancement tester (ENH- Bart enhancer) which holds 15mL of sample which is then rotated for one minute at room temperature ($22\pm2^{o}C$) to trigger an intensification of the bacteriological activity. Rotation is achieved for the tester by rotating the tester for 3 rpm in one minute. Rotating the sample triggers metabolic activity with the stimulated storage of ATP. Once rotation has been completed then a mid-point sampling now is used to start the standard total ATP analysis. The protocol for this testing is defined below and generates ATP values as E-tATP commonly with three to ten times higher than the standard tests with better precision.

### 14.3 Provisional Protocol to determine the E-tATP concentration in liquid samples

Take 15 ml liquid sample for E-tATP testing and charge the ENH- enhancer. The charged and sealed enhancer is now rotated at 3 rpm for one minute. During the rotation sequence the sample moves six times from one end of the enhancer to the other with the ball rolling along the tube and causing turbulence and agitation (maintaining oxygen saturation in the ENH- enhancer). At the same time the crystallised selective medium pellet in the base of the enhancer rapidly begins to dissolve and provides additional organics to enhance the bacteriological activity. Immediately (within five minutes) of when the rotation is finished then a 1ml midpoint sample is taken to conduct a total ATP measurement using procedure described below This is done using the following steps:

a) Unscrew the cap and placing it upside down on a clean surface,
b) Push the ball to the bottom of the enhancer tube using the micropippettor set at 1mL,
c) Remove 1ml as a midpoint sample using the micropippettor,
d) Conducting the E-tATP test using procedure set out below
e) Screw down the cap back onto the tester prior to disposal following recommended procedures.

Note that latex gloves should be worn during this procedure and any surfaces disinfected using standard practices. There are two stages in the analysis of a sample for E-tATP. The first stage relates to the calibration of a standard from the luminase for use during all of the tests being conducted within four hours. In the second stage the sample is analysed for ATP using the luminase methodology. Data is generated primarily as relative light (luciferase) units (RLU) and

these (calibration and test sample data) are then converted into E-tATP using a standard equation.

## 14.4 Procedure for the Generation of RLU values for the E-tATP

There are two steps in the determination of the E-tATP involving calibration of the Luminase (step one) and then using that calibrated Luminase to determine the E-tATP (as step two).In both steps the data product is given in RLU (relative luciferase / light units) with the E-tATP only being calculated when both steps are finished.

### 14.4.1 Calibration

Calibration of the Luminase consists of three procedures performed quickly one after the other. They are:

- Pipette 100uL of luminase into a 12x55mm Polypropylene Culture Tube
- Add 100uL of UltraCheck to the tube
- Gently provide a wrist action shake the tube for three seconds
- Immediately place the tube into the PhotonMaster luminometer
- Press “START”
- Wait for ten seconds until the RLU number is displayed
- Write down the calibration number ($RLU/_{UCL}$).

It is recommended that precision is improved if this step one calibration is repeated twice more to give three $RLU/_{UCL}$ numbers. When this is done then the $RLU/_{UCL}$ number used would be the average of the three values. This RLU number should be greater than 5,000 and the variance should be less than 5%.

### 14.4.2 Determination of the RLU from an Active Sample that has been subjected to Enhancement (E-tATP)

This method utilizes 1ml of midpoint sample taken after the agitation of the wastewater sample for one minute at 3rpm suing the STS- enhancer. There are nine manipulations involved in the protocol for RLU for the enhanced sample:

- Add 1ml of UltraLyse7 to a 17 x 100mm extraction tube.
- Add 9ml of UltraLute to a second 17 x 100mm extraction tube.
- Dispense 100microliters to an 12 x 55mm assay tube. (Note that the Luminase needs to be removed from the freezer for at least one hour before ATP testing to ensure that the Luminase acclimatises to room temperature).
- Agitate the sample to be tested and remove 1ml and place in the extraction tube that already has the Ultralyse7. Cap the tube, mix and then hold for five minutes using the electronic timer to allow the reaction to proceed.
- Take a midpoint sample and transfer to the extraction tube containing UltraLute, cap and mix by agitation.
- Immediately withdraw from the mid-point of the extraction tube 100microliters and add to the assay tube containing the luciferase.
- Place assay tube quickly into the PhotonMaster luminometer and wait ten seconds or until a number appears on the screen of the luminometer which is now used to calculate the RLU.
- The $RLU_S$ value is generated for the agitated (stressed) sample.
- ATP value can now be calculated (see section 5, protocol b)

### 14.4.2 Calculation Protocol for the Determination of the E-tATP

Data used to calculate the E-tATP includes the calibration $RLU/_{UCL}$ and the stressed sample under test as $RLU/_S$. The equation is listed below for the calculation of the E-tATP in pg/mL. Where a sufficient number (>8) of tests are being performed for the E-tATP then it is recommended that the $RLU/_{UCL}$ be calculated as the average of three replicated readings on the Luminase to improve precision. It should be noted that once calibrated that sample of Luminase can be used (at room temperature) for four hours before it begins to significantly degenerate.

$$\text{E-tATP} = (\text{RLUs}/\text{RLU}_{\text{UCI}}) \times 20{,}000$$

E-tATP is calculated in picograms per ml (pg / ml), tATP is calculated using the RLUs which, along with $RLU_{UCI}$, generates the E-tATP number. Relationship of the E-tATP to the BOD is summarized in Table 14.1 below.

### 14.5. Relationship of generated E-tATP value to Biochemical Oxygen Demand

For those (125) samples generating E-tATP values of less than 10,000pg/mL all had acceptably low BOD values (<15 mg/L). Between 10,000 and 20,000pg/mL there were only 27 samples and all gave acceptable BOD values of <25mg/L. Data from 20,000 to <40,000pg/mL suggested that there would be a high probability (67%) that the sample would have a value exceeding BOD of 25 and would require confirmatory testing and further treatment before environmentally suitable discharge. Within the 40,000 to 80,000 range investigated only 3% originated from final effluent with a low BOD (<25mg/L). From this data it was evident that for the samples below 20,000pg/mL (41% of the total count of 217 samples). only one related to a PI sample while the rest were all CL or FE with acceptable BOD values for discharge. The one outlier (0.6% of the samples found within this range) generated less than 20,000pg/mL which represented a very low percentage of failure (false negative). On the basis of the above critical examination of 217 samples it was found, with all BOD tests being performed using the carbonaceous biochemical oxygen demand (CBOD) test method. It was found that there was a quantitative link between the E-tATP values (of less than 20,000pg/mL) and the CBOD using equation one. Here the equation generates a predicted biochemical; oxygen demand (PBOD) value For E-tATP values in excess of 20,000pg/mL it was found there was a “grey” zone between 20,000 and 40,000pg/mL when two thirds of the sample indicated additional treatment would be required to bring the CBOD down to regulatory requirements. Over 40,000pg/mL there remained extensive oxygen demands that would not allow effective environmentally safe discharge. Equation one therefore specifically addresses the range below 20,000pg/mL and should not be applied to those samples having greater than 20,000pg/mL. Values in excess of 20,000pg/mL should be considered as having too high a remaining biochemical oxygen demand to allow a safe and regulated discharge. Refer to the thirty hour percentage confirmatory bacteriological reduction (%CBR) test for confirmation of acceptable BOD discharge levels.

$$PBOD = \text{E-tATP} / 800$$

Where PBOD is the predicted biochemical oxygen demand in mg/L; E-tATP is the test value obtained for the sample that has to be 20,000pg/mL or less; and 800 is the standard correction factor applicable only over the specified range from 1 to 20,000pg/mL.

Table 14.1 gives the provisional relationship between the E-tATP (in pg/mL) generated in fifteen minutes and the predicted biochemical oxygen demand (PBOD) which is claimed to be comparable to the CBOD. This is based upon equation one above and may only be applied to samples generating 20,000pg/mL or less with those samples having more than 20,000pg/mL requiring confirmatory testing using the %CBR thirty hour (maximum).

**Table 14.1 Probable relationship of E-tATP values in pg/mL to PBOD generated using Equation one**

| E-tATP pg/mL | PBOD mg/L |
|---|---|
| **20,000** | **25.0** |
| **19,000** | **23.8** |
| **18,000** | **22.5** |
| **17,000** | **21.3** |
| **16,000** | **20.0** |
| **15,000** | **18.8** |
| **14,000** | **17.5** |
| **13,000** | **16.3** |
| **12,000** | **15.0** |
| **11,000** | **13.8** |
| **10,000** | **12.5** |
| **9,000** | **11.3** |
| **8,000** | **10.0** |
| **7,000** | **8.8** |
| **6,000** | **7.5** |
| **5,000** | **6.3** |
| **4,000** | **5.0** |
| **3,000** | **3.8** |
| **2,000** | **2.5** |
| **1,000** | **1.3** |

## 14.6 Rapid BOD ATP test system, requirements

### 14.6.1 Parts to complete a Total ATP test:

From: Luminultra Technologies Ltd., Fredericton

1. UltraLute, ULU requires 9ml per test
2. Ultralyse 7, UL7 requires 1ml per test
3. Luminase, LU requires 100microL per test (plus 100microL for calibration)
4. 12x55mm Polypropylene Culture Tubes requires 200microL per test
5. 17x100mm Polypropylene Culture Tubes
6. Ultracheck1, UC1 requires 100microL for calibration
7. 17mm Polypropylene Culture Tube Caps
8. Pipet tips 20 – 200 μL PT1
9. Pipet tips 0.1 – 1.0ml PT1
10. Pipet tips 1.0 – 5ml PT5
11. PhotonMaster Luminometer (EQP-PMT)
12. 1-5mL Adjustable Micropipettor
13. 100-1000μL Adjustable Micropipettor
14. 100-200 μL Adjustable Micropipettor

From: Droycon Bioconcepts Inc., Regina

15. ENH- BART laboratory tester, beige flexible capped SRTS-BART (lab)
16. Stop watch
17. Model 24 Rotator 6123-29 (Reliable Scientific, Inc. Nesbit, MS) plus Rotator Disk 6/23-29

Note that in 2013 Luminultra changed the technology for the total ATP test with a new protocol: ”QuenchGone 21 Industrial (QG21I)”. Note that there is a different formulation for calculating the total ATP and this would need to be used for calculating the E-tATP. The provisional protocol is based upon the original E-tATP as described in this chapter.

## 14.7 Provisional Protocol, Rapid BOD (ATP)

Take 15 ml sample for rapid BOD testing and use 1ml midpoint sample is taken to conduct a total ATP measurement using procedure that begins with taking 15ml of the sample which is now immediately added to the ENH- BART (lab) tester and capped. Note that 15ml of sample takes the fluid up to within 1mm of the fill line. The charged tester is now clipped into the rotator agitated for one minute using the rotator set at 3rpm. This is performed at room temperature. Immediately after the end of the one minute agitation period at 3 rpm then 1ml of the sample is now taken from the midpoint of the STRS- BART tester. This is done using the following steps:

a) unscrewing the cap and placing it upside down on a clean surface,
b) pushing the ball to the bottom of the tube using the micropippettor set at 1mL,
c) removing 1ml as a midpoint sample,
d) conducting the total ATP test using procedure (a) set out in 3screw down the cap back onto the tester prior to disposal following recommended procedures.

Note that latex gloves should be worn during this procedure and any surfaces disinfected using standard practices. From the two rapid BOD tests following should be two numbers generated as relative light (luciferase) units (RLU).

## 14.8 Procedure (a) Generation of RLU values

This method utilizes 1ml of sample taken after the agitation of the sample. The nature of the test is described as bullets below:

- Add 1ml of UltraLyse7 to a 17 x 100mm extraction tube.
- Add 9ml of UltraLute to a second 17 x 100mm extraction tube.
- Dispense 100microliters to an assay tube. (Note that the Luminase needs to be removed from the refrigerator one hour before ATP testing so that the Luminase has acclimatised to room temperature).
- Agitate the sample to be tested and remove 1ml and place in the extraction tube that already has the Ultralyse7. Cap the tube, mix and then hold for five minutes.
- Take a midpoint sample and transfer to the extraction tube containing UltraLute, cap and mix by agitation.
- Immediately withdraw from the mid-point of the extraction tube 100microliters and add to the assay tube containing the luciferase.

- Place assay tube quickly into the PhotonMaster luminometer and wait ten seconds or until a number appears on the screen of the luminometer which is now used to calculate the RLU.
- The RLU value is generated for the agitated (stressed) sample.
- ATP value can now be calculated (see section 4, protocol b)

### 14.9 Protocol b, calculation of ATP

To calculate the enhanced total ATP (E-tATP) it is necessary to obtain a basic RLU value for the calibration of the strength of the Luminase that has been stored in the refrigerator but returned to room temperature before use. To calibrate the Luminase then two drops (100microliters) of Luminase is mixed with two drops of UltracheckI in an assay tube and immediately placed in the luminometer to obtain $RLU_{UC1}$. Calculating the E-tATP for the test (RLUs) involves the same formula:

$$\textbf{E-tATP} = \textbf{(RLUs/RLU}_{\textbf{UCI}}\textbf{)} \times \textbf{20,000}$$

E-tATP is calculated in picograms per ml (pg / ml), tATP is calculated using the RLUs which, along with $RLU_{UCI}$, generates the E-tATP. Note that QuenchGone 21 uses a standard correction factor of 10,000 rather than 20,000 described in 14.9.

### 14.10 E-tATP interpretation in Bart testing

E-tATP is one measure of bacterial activity in a sample that can be used to decide whether testing using the Bart testers would be likely to deliver positive detections. In the DBI laboratories it is now a routine practise to precede Bart testing with measurement of the E-tATP activity levels in the sample under investigation. The concept here would simply one of economy. If the E-tATP exhibits little or no activity then that means there is either very few active cells in the samples or that the samples are in a passive mode (i.e. suspended animation). With the first alternative the Bart testers will not be able to detect what is not there. For the second alternative then the cells are passive (not active). It is very possible that at least some of the cells will be triggered by the conditions in the Bart tester to become active which would cause extended time lapses before the onset of the growth phase. In practise it has been found in the DBI laboratories the when the E-tATP is measured and found to be less than 50pg/mL there is little likelihood that the Bart testers will respond quickly and detect these bacteria. If the E-

tATP is over 100pg/mL then this would indicate that there was some activity and Bart testing would be worthwhile. It would be reasonable to set the minimum E-tATP at 100pg/mL if the Bart testing was routine. However in those events where there needs to be confirmation of very low bacterial populations and activity then the start point for Bart testing could be lowered to 50pg/mL. As E-tATP values rise above 100 to 1,000pg/mL then so there is a greater certainty that Bart testing will yield positive detections. For E-tATP values higher than 1,000pg/mL then there is a virtual certainty that active bacteria are present (at the base of the food chain) and that higher plants and animals may now thrive if conditions are oxidative. Reductive conditions are likely to remain dominated by the anaerobic fermentative bacteria that would now dominate the communities. As E-tATP values become higher (e.g. 10,000 and 100,000pg/mL) then there is an increasing certainty the Bart testing will trigger very active populations.