FINAL REPORT
DRA-8

Marine aerobic biodegradation test of NuPlastiQ GP 1000

Author: Lynn Serbruyns
Sponsor: BiologiQ, Inc.
2400 East 25th St
Idaho Falls
ID 83404
UNITED STATES
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1 Identification of the test

1.1 General information

Project number
DRA-8

Sponsor
BiologiQ, Inc.
2400 East 25th St
Idaho Falls
ID 83404
UNITED STATES

Sponsor contact
Dr. Don Allen
Phone: +1 208 357 9653
Don.Allen@BiologiQ.com

Testing facility
OWS nv
Dok-Noord 5
9000 Gent
BELGIUM
Phone: +32 9 233 02 04
Fax: +32 9 233 28 25
bruno.dewilde@ows.be
lynn.serbruyns@ows.be

Contact in the United States
Mr. Richard Tillinger
Phone: +1 937 829 6888
richard.tillinger@ows.be

Mrs. Norma McDonald
Phone: +1 513 535 6760
norma.mcdonald@ows.be

Test item
NuPlastiQ GP 1000

Reference item
Cellulose

Test duration
28 days
1.2 Study personnel

Study Director: Lynn Serbruyns
Replacement Study Director: Wouter Thys
Study Director QA: Johan Vermeulen

1.3 Study schedule

Study initiation date: March 29th, 2018
Experimental starting date: April 4th, 2018
Starting date of incubation: April 4th, 2018
Completion date of incubation: May 2nd, 2018
Experimental completion date: May 8th, 2018
Study completion date: May 22nd, 2018
Total test duration: 28 days

1.4 Archiving

All raw data and records necessary to reconstruct the study and demonstrate adherence to the study plan will be maintained in the archives of OWS nv. These records include notebooks, study plan, study report, samples of test item and specimens. They will be stored in a file coded:

DRA-8

The training records of personnel are stored in the maps ‘Organisation and Personnel’. These files are stored per person and administered by the Lab Quality Manager and the Assistant Lab Quality Manager.

After seven (7) years, all data and records will be destroyed or returned to the Sponsor after agreement in writing by the involved Sponsor and the Study Director. In case no written agreement of the Sponsor can be obtained after seven years, the data and records will be destroyed.
2 Confidentiality statement
The Testing Facility will treat strictly confidential all relevant information on the test item disclosed by the Sponsor as well as all results obtained in executing the test.

___________________
Bruno De Wilde
Lab Manager

3 GLP compliance statement
The test was performed in accordance with the OECD principles of Good Laboratory Practices (GLP).

___________________
Lynn Serbruyns
Study Director

4 Quality assurance audit statement
The results reported are in accordance with the study plan and raw data.

A quality control was executed on ..May:23:2018

This quality control ensures that the final report is complete and accurately reflects the conduct and raw data of the study.

___________________
Johan Vermeulen
Study Director QA
5 Summary and conclusions

The aerobic biodegradation of test item NuPlastiQ GP 1000 was evaluated in a marine biodegradation test according to ASTM D6691 (2017). The test was performed in triplicate and the incubation temperature was continuously kept at 30°C ± 2°C. The total test duration was 28 days and the final biodegradation percentages are based on CO₂ production.

According to the ASTM D6691 (2017) standard, the test is considered valid if the degree of biodegradation of the reference material is > 70% at the end of the test. The results show clearly that the requirement was fulfilled. After 28 days a biodegradation percentage of 84.4% ± 0.4% was measured for reference item cellulose.

The biodegradation of test item NuPlastiQ GP 1000 proceeded well throughout the test. After 28 days (end of test) an absolute biodegradation of 92.4% ± 1.6% was measured. On a relative basis, compared to suitable reference substrate cellulose, a biodegradation percentage of 109.5% was calculated.

According to the OK Biodegradable MARINE certification scheme of TÜV AUSTRIA Belgium, a material can only be called biodegradable when the percentage of biodegradation of a test material is at least 90% in total or 90% of the maximum degradation of a suitable reference substrate after a plateau has been reached for both test material and reference substance. The maximum allowed test duration is 6 months. From these results it can be concluded that test item NuPlastiQ GP 1000 is completely biodegradable within 28 days of testing under marine aerobic conditions.
6 Introduction

6.1 Principle of test method

The marine biodegradation test determines the biodegradation of a test item under laboratory conditions by incubation in seawater. The test material is brought into natural seawater enriched with inorganic nutrients (0.05 g/l of NH₄Cl and 0.1 g/l of KH₂PO₄) and containing an indigenous population of micro-organisms.

During the aerobic biodegradation of organic materials in a marine medium, oxygen is consumed and carbon is converted to gaseous, mineral C (under the form of carbon dioxide, CO₂). Part of the organic material is assimilated for cell growth. KOH solution traps the CO₂ released and the induced pressure-drop is directly related to the consumed oxygen and hence to the biodegradation of the test item.

The amount of biodegradation based on O₂ consumption is expressed as the ratio of the BOD (corrected for the control) to the Theoretical Oxygen Demand (ThOD) or Chemical Oxygen Demand (COD) of the used test item. The biodegradation based on CO₂ production is calculated as the percentage of solid carbon of the test compound which has been converted to gaseous, mineral C under the form of CO₂.

6.2 Standard followed

7 Materials and methods

7.1 Test and reference item

Test item
Name: NuPlastiQ GP 1000
Description: Pellets
Colour: Light yellow transparent
Batch number: #506
Production date: Feb-16-2018
Production process: EcoLogiQ (proprietary)
Sample preparation: Cryogenically milled (< 1 mm)

Reference item
Name: Cellulose
Purity: Native cellulose powder for thin layer chromatography (Avicel)
Physical form: Powder
Colour: White
Batch number: K45989631508
Expiration date: February 2021

7.2 General procedure

The source of micro-organisms (inoculum) is a 1:1 mixture of coastal seawater and seawater sampled from open sea (both from the Belgian part of the North Sea). The seawater is sieved on a 80 µm screen and enriched with inorganic nutrients (0.05 g/l of NH₄Cl and 0.1 g/l of KH₂PO₄) prior to use.

At the start of the test, each reactor is filled with the same amount of enriched seawater. The reference and test item are added directly to the reactors. After filling of the reactors, KOH solution is added to the rubber carriers, OXITOP-OC heads are connected and the reactors are put on an inductive stirrer (see Figure 1). A magnetic rod keeps the reference item, test item and the growing biomass into suspension throughout the test. The vessels are aerated for 15 minutes in the incubator before closing and initiating the actual incubation period for biodegradation. This final aeration period is needed to equilibrate the final mixture and to stabilize the temperature. The reactors are incubated at a constant temperature (30°C ± 2°C) in the dark for a period of minimum 28 days.

During the test, the KOH solution absorbs the CO₂ produced. This absorption causes a pressure drop inside the reactors which can be translated to a given O₂ consumption. The Biological Oxygen Demand (BOD) is continuously analysed on regular intervals (every 3 hours). The percentage of biodegradation based on O₂ consumption is expressed as the ratio of the BOD (corrected for the control) to the Theoretical Oxygen Demand (ThOD) or Chemical Oxygen Demand (COD) of the used test item.

At regular intervals (every two weeks) the amount of CO₂ produced is determined by titration of the KOH solution. The biodegradation based on CO₂ production is calculated as the percentage of solid carbon of the test compound which has been converted to gaseous, mineral C under the form of CO₂.
7.3 Analytical methods

**Ammonium - nitrogen (NH₄⁺-N)**

This analysis is done as described in ‘M_016. Determination of ammonia-nitrogen by FIA (spectrometric detection)’. The determination is performed on the aqueous sample after filtration through a syringe filter (pore size = 1.20 µm). The sample containing ammonium ions is injected into a continuous carrier stream by means of an injection valve and is mixed with a continuously streaming flow of an alkaline solution. The gaseous ammonia formed is separated through a diffusion cell from the solution over a hydrophobic semi permeable membrane and taken up by a streaming recipient flow containing a pH indicator. Due to the resulting pH shift, the indicator solution will change its colour which is measured continuously in the flow photometer at 590 nm. The results are given in g per l wet weight.

**Dry matter or total solids (TS)**

The dry matter is determined by drying at 105°C for at least 14 hours and weighing, as described in ‘M_009. Determination of moisture content’. The dry matter is given in percent on wet weight.

**Elemental analysis CHN**

The elemental analysis CHN is determined in an external lab and is conducted according to DIN 51732 (2014). The results are expressed in per cent.
**Kjeldahl nitrogen (Kj-N)**

This analysis is done as described in 'M_036. Determination of Kjeldahl nitrogen'. In the presence of a catalysing agent (K$_2$SO$_4$-Se-mixture) and under boiling conditions (380°C) with a mixture of sulphuric acid bound nitrogen is converted into the salt (NH$_4$)$_2$SO$_4$. Afterwards the ammonia is liberated using strong alkali and distilled for subsequent determination by titration. The ammonia is captured in a boric acid/indicator solution. Determination of ammonium ion in the distillate is done by titration with standard acid. The results are given in g per kg total solids.

**Nitrate and nitrite - nitrogen (NO$_x$-N)**

This analysis is done as described in 'M_017. Determination of total oxidized nitrogen by FIA (spectrometric detection)’. The determination is performed on the aqueous sample after filtration through a syringe filter (pore size = 1.20 µm). The sample containing nitrite/nitrate ions is fed into a continuously flowing buffer solution (carrier stream) by means of an injection valve. Nitrate in the sample is reduced to nitrite in a cadmium reductor. On the addition of an acidic sulphanilamide solution, nitrite initially present and nitrite formed from reduction of nitrate will form a diazo compound. This compound is coupled with N-(1-naphthyl)-ethylene diamine dihydrochloride (NED) to form a purple azo dye. This azo dye is measured at 540 nm. The results are given in g per l wet weight.

**pH**

The pH is measured directly on the aqueous sample with a pH meter after calibration with standard buffer solutions (pH = 4.0, pH = 7.0 and pH = 10.0), as described in 'M_006. Determination of pH and electrical conductivity’.

**Theoretical oxygen demand (ThOD)**

The ThOD is calculated from the chemical formula or based on the elemental analysis (which is determined in another lab) of the test material, according to the formula described in ISO 14851 (2005), Annex A. The results are given in g/g.

**Titration**

The amount of CO$_2$ captured in a 3N KOH solution (with the formation of K$_2$CO$_3$), is determined titrimetrically with 0.05N HCl. The titre of HCl is determined with a 0.05N KOH solution. The titration is done in two steps with an automatic titrator (Metrohm 888 Titrando). The first step involves the conversion of the excess of KOH to KCl and of K$_2$CO$_3$ to KHCO$_3$ (pH = 8.0). The second step involves the conversion of KHCO$_3$ to KCl and CO$_2$ (pH = 3.8). The results are given in ml. The amount of HCl used during the second titration step is a direct measure for the amount of CO$_2$ which is captured (1 meq HCl titrated = 1 meq CO$_2$ captured).

**Total organic carbon (TOC)**

The total organic carbon content is determined by subtracting the total inorganic carbon content from the total carbon content as described in 'M_039. Determination of total organic carbon and total nitrogen – method by total carbon, total nitrogen and inorganic carbon combustion’. The total carbon content is determined using a high temperature (950°C to 1200°C) combustion method. The formed CO$_2$ is measured with IR detection. Inorganic carbon is measured by acidification of the sample and heating at 150°C. The released CO$_2$ is determined by IR detection. The results are given in percent on wet weight.
Volatile solids (VS) - ash
The volatile solids and ash content is determined by heating the dried sample at 550°C for at least 4 hours and weighing, as described in ‘M_010. Determination of organic matter and carbon content’. The results are given in percent on dry matter.

Weight determination
During the test 3 types of balances are used. A Sartorius AC 210 S with internal calibration (max. 200 g, d = 0.1 mg) for the determination of dry and volatile matter. A Sartorius AX224 (max. 220 g; d = 0.1 mg) for weighing of the test and reference item. A Sartorius ED 3202S-CW (max. 3,200 g; d = 0.01 g) or a Acculab ATL-4202 (max. 4,200 g, d = 0.01 g) for weighing of the inoculum and mineral medium.
8 Results

8.1 Test conditions and set-up

A set of 9 equal reactor vessels with a total volume of 500 ml each was used. The reactors were filled with 250 g of enriched seawater. Reference item cellulose was added as powder, while test item NuPlastiQ GP 1000 was previously reduced in size (cryogenically milled until < 1 mm). The test set-up is given in Table 1. After the addition of the reference and test item, the reactors were put on an inductive stirrer. A magnetic rod kept the reference item, test item and growing biomass into suspension throughout the test. The reactors were incubated at a constant temperature of 30°C ± 2°C in the dark. The total test duration was 28 days.

<table>
<thead>
<tr>
<th>RN</th>
<th>Test series</th>
<th>Enriched seawater (g)</th>
<th>Item (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Cellulose</td>
<td>250</td>
<td>59.9</td>
</tr>
<tr>
<td>5</td>
<td>Cellulose</td>
<td>250</td>
<td>60.0</td>
</tr>
<tr>
<td>6</td>
<td>Cellulose</td>
<td>250</td>
<td>59.9</td>
</tr>
<tr>
<td>7</td>
<td>NuPlastiQ GP 1000</td>
<td>250</td>
<td>60.0</td>
</tr>
<tr>
<td>8</td>
<td>NuPlastiQ GP 1000</td>
<td>250</td>
<td>59.9</td>
</tr>
<tr>
<td>9</td>
<td>NuPlastiQ GP 1000</td>
<td>250</td>
<td>60.0</td>
</tr>
</tbody>
</table>

RN = reactor number

8.2 Analyses of inoculum, test and reference item

The inoculum was a 1:1 mixture of coastal seawater and seawater sampled from open sea (both from the Belgian part of the North Sea). After filtration over an 80 µm sieve, inorganic nutrients were added to the seawater in a concentration of 0.05 g/l NH₄Cl and 0.1 g/l KH₂PO₄. The total solids (TS), volatile solids (VS), pH and nitrogen analyses of the inoculum are given in Table 2.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (TS, %)</td>
<td>3.3</td>
</tr>
<tr>
<td>Volatile solids (VS, % on TS)</td>
<td>13.3</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
<tr>
<td>Kjeldahl-N (mg/l)</td>
<td>34</td>
</tr>
<tr>
<td>NH₄⁺ -N (mg/l)</td>
<td>26.4</td>
</tr>
<tr>
<td>NO₃⁻ -N (mg/l)</td>
<td>2.6</td>
</tr>
</tbody>
</table>

The total solids (TS), volatile solids (VS), theoretical oxygen demand (ThOD, calculated from elemental analysis) and total organic carbon content (TOC) of the reference and test item are summarised in Table 3.

<table>
<thead>
<tr>
<th>Test item</th>
<th>TS (%)</th>
<th>VS (% on TS)</th>
<th>ThOD (mg/g)</th>
<th>TOC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>97.0</td>
<td>100.0</td>
<td>1212</td>
<td>42.7</td>
</tr>
<tr>
<td>NuPlastiQ GP 1000</td>
<td>85.8</td>
<td>99.8</td>
<td>1082</td>
<td>38.3</td>
</tr>
</tbody>
</table>
8.3 Biodegradation percentages

8.3.1 Biodegradation based on O$_2$ consumption

Biodegradation was determined by measuring the amount of O$_2$ consumption throughout the test. The calculation of the biodegradation percentages is based on the net oxygen consumption (after subtraction of the oxygen consumed in the control reactors) in the test or reference reactor and on the ThOD added to each reactor. At the end of the test (28 days) all vessels were checked on the presence of nitrate and nitrite by means of nitrate/nitrite strips. Small amounts of nitrate and nitrite were found in the control and cellulose reactors (see Table 4). As a result a correction for oxygen consumption by nitrification was made.

<table>
<thead>
<tr>
<th>RN</th>
<th>Test series</th>
<th>pH</th>
<th>NH$_4^+$-N (mg/l)</th>
<th>NO$_3^-$-N (mg/l)</th>
<th>NO$_2^-$-N (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>8.7</td>
<td>22.8</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>8.7</td>
<td>22.5</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>8.7</td>
<td>21.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>Cellulose</td>
<td>8.4</td>
<td>16.7</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>Cellulose</td>
<td>8.5</td>
<td>18.8</td>
<td>1.1</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>Cellulose</td>
<td>8.4</td>
<td>19.8</td>
<td>1.2</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>NuPlastiQ GP 1000</td>
<td>8.7</td>
<td>20.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>NuPlastiQ GP 1000</td>
<td>8.7</td>
<td>17.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>NuPlastiQ GP 1000</td>
<td>8.7</td>
<td>20.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 4. pH and nitrogen analyses at end of test (28 days)

Table 5 shows the ThOD (theoretical oxygen demand), net O$_2$ consumption and biodegradation percentage of reference and test item at the end of the test (28 days). The evolution of the cumulative O$_2$ consumption of the control, reference and test item is represented in Figures 2 up to 4. Figure 5 shows the evolution of the average biodegradation of reference and test item (based on O$_2$ consumption), while Figures 6 and 7 show the biodegradation of the replicates.

<table>
<thead>
<tr>
<th>Test series</th>
<th>Before correction</th>
<th>After correction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Net O$_2$</td>
<td>Biodegradation (%)</td>
</tr>
<tr>
<td></td>
<td>AVG</td>
<td>SD</td>
</tr>
<tr>
<td>Cellulose</td>
<td>237.8</td>
<td>81.8</td>
</tr>
<tr>
<td>NuPlastiQ GP 1000</td>
<td>243.1</td>
<td>93.7</td>
</tr>
</tbody>
</table>

Table 5. Biodegradation based on O$_2$ consumption at end of test (28 days) before and after correction for nitrification

With AVG = average, SD = standard deviation and REL = relative biodegradation.

The biodegradation of reference item cellulose started at a good rate after a lag phase of approximately 1 day. After 4 days cellulose was already degraded by 55.1%. From then on biodegradation rate gradually slowed down and after 28 days (end of test) a plateau was reached at an absolute, corrected biodegradation of 80.9% ± 2.3%. The test is considered valid if the biodegradation percentage of the reference item is more than 70% at the end of the test. This requirement was fulfilled.

The biodegradation of test item NuPlastiQ GP 1000 started more or less at the same time as reference item cellulose, but proceeded even faster. After 4 days NuPlastiQ GP 1000 was already degraded by 76.7%. At the end of the test (28 days) a plateau was reached at an absolute, corrected biodegradation of 96.9% ± 2.0%. On a relative basis, compared to suitable reference substrate cellulose, a biodegradation percentage of 119.7% was calculated.
Figure 2. Evolution of the cumulative $O_2$ consumption of the control reactors

Figure 3. Evolution of the cumulative $O_2$ consumption of the cellulose reactors
Figure 4. Evolution of the cumulative $O_2$ consumption of the NuPlastiQ GP 1000 reactors

Figure 5. Evolution of the average biodegradation percentage of reference and test item (based on $O_2$ consumption)
Figure 6. Evolution of the biodegradation percentage of replicates of cellulose (based on $O_2$ consumption)

Figure 7. Evolution of the biodegradation percentage of replicates of NuPlastiQ GP 1000 (based on $O_2$ consumption)
8.3.2 Biodegradation based on CO₂ production

The biodegradation was also determined by measuring the amount of CO₂ that had been captured in the KOH solution during the test.

Table 6 shows the ThCO₂ (= theoretical CO₂ production based on the % organic C and input of the sample), net CO₂ production and percentage of reference and test item at the end of the test (28 days). A visual presentation of the cumulative CO₂ production of the control, reference and test item is given in Figures 8 up to 10. Figure 11 shows the evolution of the average biodegradation of reference and test item (based on CO₂ production), while Figures 12 and 13 show the biodegradation of the replicates.

Table 6. TOC, net CO₂ production and biodegradation after 28 days

<table>
<thead>
<tr>
<th>Test series</th>
<th>ThCO₂ (mg)</th>
<th>Net CO₂ (mg)</th>
<th>Biodegradation (%)</th>
<th>95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>93.7</td>
<td>79.1</td>
<td>84.4</td>
<td>0.4</td>
</tr>
<tr>
<td>NuPlastiQ GP 1000</td>
<td>84.2</td>
<td>77.8</td>
<td>92.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

With AVG = average, SD = standard deviation, REL = relative biodegradation and CL = confidence limits

The test is considered valid, if at the end of the test the biodegradation percentage of the reference item cellulose is more than 70%. After 28 days a plateau in biodegradation was reached at a level of 84.4% ± 0.4%. The requirement was clearly fulfilled.

The biodegradation of test item NuPlastiQ GP 1000 proceeded well throughout the test. After 28 days (end of test) an absolute biodegradation of 92.4% ± 1.6% was measured. On a relative basis, compared to suitable reference substrate cellulose, a biodegradation percentage of 109.5% was calculated.

According to the OK Biodegradable MARINE certification scheme of TÜV AUSTRIA Belgium, a material can only be called biodegradable when the percentage of biodegradation of a test material is at least 90% in total or 90% of the maximum degradation of a suitable reference substrate after a plateau has been reached for both test material and reference substance. The maximum allowed test duration is 6 months. From these results it can be concluded that test item NuPlastiQ GP 1000 is completely biodegradable within 28 days of testing under marine aerobic conditions.
Figure 8. Evolution of the cumulative CO$_2$ production of the control reactors

Figure 9. Evolution of the cumulative CO$_2$ production of the cellulose reactors
Figure 10. Evolution of the cumulative CO₂ production of the NuPlastiQ GP 1000 reactors

Figure 11. Evolution of the average biodegradation percentage of reference and test item (based on CO₂ production)
Figure 12. Evolution of the biodegradation percentage of replicates of cellulose (based on $\text{CO}_2$ production)

Figure 13. Evolution of the biodegradation percentage of replicates of NuPlastiQ GP 1000 (based on $\text{CO}_2$ production)