# FINAL REPORT
# GVR-1

## *Aqueous aerobic biodegradation test on* ***CF-7***

**Author:** Sylvie Denis
**Sponsor:** C-Fire
Frank Van Dyckelaan 17
9140 Temse
BELGIUM

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# 1 Identification of the test

## 1.1 General information

### Project number

GVR-1

### Sponsor

C-Fire
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### Sponsor contact

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### Reference item

Sodium acetate

### Test item

CF-7

### Test duration

28 days

## 1.2 Study personnel

| | |
|---|---|
| Study Director: | Sylvie Denis |
| Replacement Study Director(s): | Eveline Beeckman<br>Joris Bril<br>Olive Nkundwakazi |
| Study Director(s) QA: | Lander De Zutter<br>Lynn Serbruyns<br>Michela Siotto<br>Wouter Thys |

## 1.3 Study schedule

| | |
|---|---|
| Starting date study: | April 3, 2023 |
| Starting date experiments: | April 3, 2023 |
| Starting date of incubation: | April 5, 2023 |
| Completion date of incubation: | May 3, 2023 |
| Test duration: | 28 days |
| Completion date of experiments: | May 15, 2023 |
| Completion date study: | May 16, 2023 |

## 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 Normec OWS nv. These records include notebooks, study plan, study report, samples of test item and specimens. They will be stored in a file coded:

GVR-1

The training records of personnel are stored in the maps 'Organisation and Personnel'. These files are stored per person and administered by the (Assistant) Quality Manager.

After seven (7) years, all data and records will be destroyed unless a written request is received from the sponsor to return it. Additives (concentration < 10% in final product) and liquids will be stored for 2 years unless a written request for storage up to 7 years is received from the sponsor.

## 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.

Lynn Serbruyns
Department Manager
Biodegradation

## 3 GLP compliance statement

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

Sylvie Denis
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 June 1, 2023.

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

Michela Siotto
Study Director QA

# 5 Summary and conclusions

The aerobic biodegradation of test item CF-7 was evaluated in an aqueous aerobic biodegradation test using sludge inoculum without any pre-adaptation to the test item according to OECD 301F (1992). The test was performed in duplicate and the incubation temperature was continuously kept at 21°C ± 1°C. The total test duration was 28 days.

According to the OECD 301F (1992) guideline, the test is considered valid if a) the degree of biodegradation of the reference material is > 60% after 14 days, and b) the oxygen consumption of the controls is not exceeding 60 mg $O_2$/l after 28 days. After 4 days Sodium acetate was already degraded by 64.7%± 0.4% (on $O_2$ consumption). The total $O_2$ consumption of the controls after 28 days of testing was 17.0 ± 2.8 mg $O_2$/l medium. Both requirements were fulfilled.

As evaluated based on oxygen consumption, the biodegradation of test item CF-7 started after approximately one day and proceeded at a good rate. After 9 days the test material was already degraded by 62.9%. The biodegradation rate gradually slowed down and at the end of the test (28 days) a biodegradation of 99.8% ± 2.4% was measured.
Based on $CO_2$ production, at the end of the test (28 days) an absolute biodegradation of 80.0% ± 2.3% was measured for test item CF-7. On a relative basis, compared to suitable reference substrate Sodium acetate, a biodegradation of 96.0% was calculated.

The test is considered valid if the difference in biodegradation between the test item replicates is less than 20% at the end of the test. After 28 days (end of test) a difference of 3.4% (on $O_2$) or 3.2% (on $CO_2$) was calculated for test item CF-7. The requirement was fulfilled.

A test item is considered to meet the biodegradation requirement of OECD *Guideline for Testing of Chemicals 301F – Manometric Respirometry Test* (1992) if 60% removal of ThOD or $ThCO_2$ is achieved. The pass level must be reached in a 10-day window within the 28-day period of the test for a chemical to be considered readily biodegradable. The 10-day window begins when the degree of biodegradation has reached 10% ThOD or $ThCO_2$ and must end before day 28 of the test. From these results it can be concluded that test item CF-7 fulfilled the 60% biodegradability requirement within 28 days of testing under the given aerobic conditions. Moreover, as the 60% pass level was reached with a 10-day window, test item CF-7 can be considered readily biodegradable.

# 6 Introduction

## 6.1 Purpose and principle of the test method

The aqueous biodegradation test determines the biodegradation of a test item under laboratory conditions by a consortium of bacteria from different sources. The test material is brought into a chemically defined (mineral) liquid medium, essentially free of other organic carbon sources, and spiked with micro-organisms.

During the aerobic biodegradation of organic materials in an aqueous medium, oxygen is consumed and carbon is converted to gaseous, mineral C (under the form of carbon dioxide, $CO_2$). Part of the organic material is assimilated for cell growth. KOH solution trap the $CO_2$ 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 $O_2$ 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 $CO_2$ 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 $CO_2$.

The test is considered as valid if:

- The degree of biodegradation of the reference material is > 60% after 14 days;
- The oxygen consumption of the controls is not exceeding 60 mg $O_2$/l after 28 days;
- The difference in biodegradation between the test item replicates is less than 20% at the end of the test.

A test item is considered to meet the biodegradation requirement if 60% removal of ThOD or $ThCO_2$ is achieved. The pass level must be reached in a 10-day window within the 28-day period of the test for a chemical to be considered readily biodegradable. The 10-day window begins when the degree of biodegradation has reached 10% ThOD or $ThCO_2$ and must end before day 28 of the test. An exception is made in the OECD *Guideline for Testing of Chemicals, Section 3* (2006) for mixtures of structurally similar chemicals (e.g. homologues of surfactants composed of fatty alcohols of varying chain length, or poly(oxyalkylene) polyol materials having defined molecular weight distributions), in which case the 10-day window does not apply.

## 6.2 Standard followed

- OECD *Guideline for Testing of Chemicals 301F – Manometric Respirometry Test* (1992).

# 7 Materials and methods

## 7.1 Reference and test item

### Reference item

| | |
|---|---|
| Name: | Sodium acetate |
| Purity: | 99%, for HPLC use |
| Physical form: | Powder |
| Colour: | White |
| CAS number: | 127-09-03 |
| Batch number: | A0435897 |
| Expiration date: | June 2027 |
| Brand: | Thermo Scientific |
| Storage conditions: | Room temperature in the dark |

### Test item

| | |
|---|---|
| Name: | CF-7 |
| Description: | Liquid |
| Colour: | Light yellow |
| Sample preparation: | None |
| Storage conditions: | Room temperature in the dark |

## 7.2 General procedure

The source of micro-organisms (inoculum) is a mixture of activated sludge, obtained from different wastewater treatment plants. The sites treat wastewater from domestic and/or industrial origin. The different sludges were sieved over an 80 µm sieve and mixed in equal parts. The final inoculum was obtained after removal of supernatant liquid and replacement with mineral medium used for the removal of soluble components, after which the inoculum is aerated for a few hours.

At the start of the test, each reactor is filled with the same amount of mineral medium. A precise amount of inoculum (1-5%) is added to each reactor to obtain a test medium with a concentration of approximately 30 mg suspended solids/l. 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 (21°C ± 1°C) in the dark for a period of minimum 28 days.

During the test, the KOH solution absorbs the $CO_2$ produced. This absorption causes a pressure drop inside the reactors which can be translated to a given $O_2$ consumption. The Biological Oxygen Demand (BOD) is continuously analysed on regular intervals (every 4 hours). The percentage of biodegradation based on $O_2$ 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 $CO_2$ produced is determined by titration of the KOH solution. The biodegradation based on $CO_2$ 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 $CO_2$.

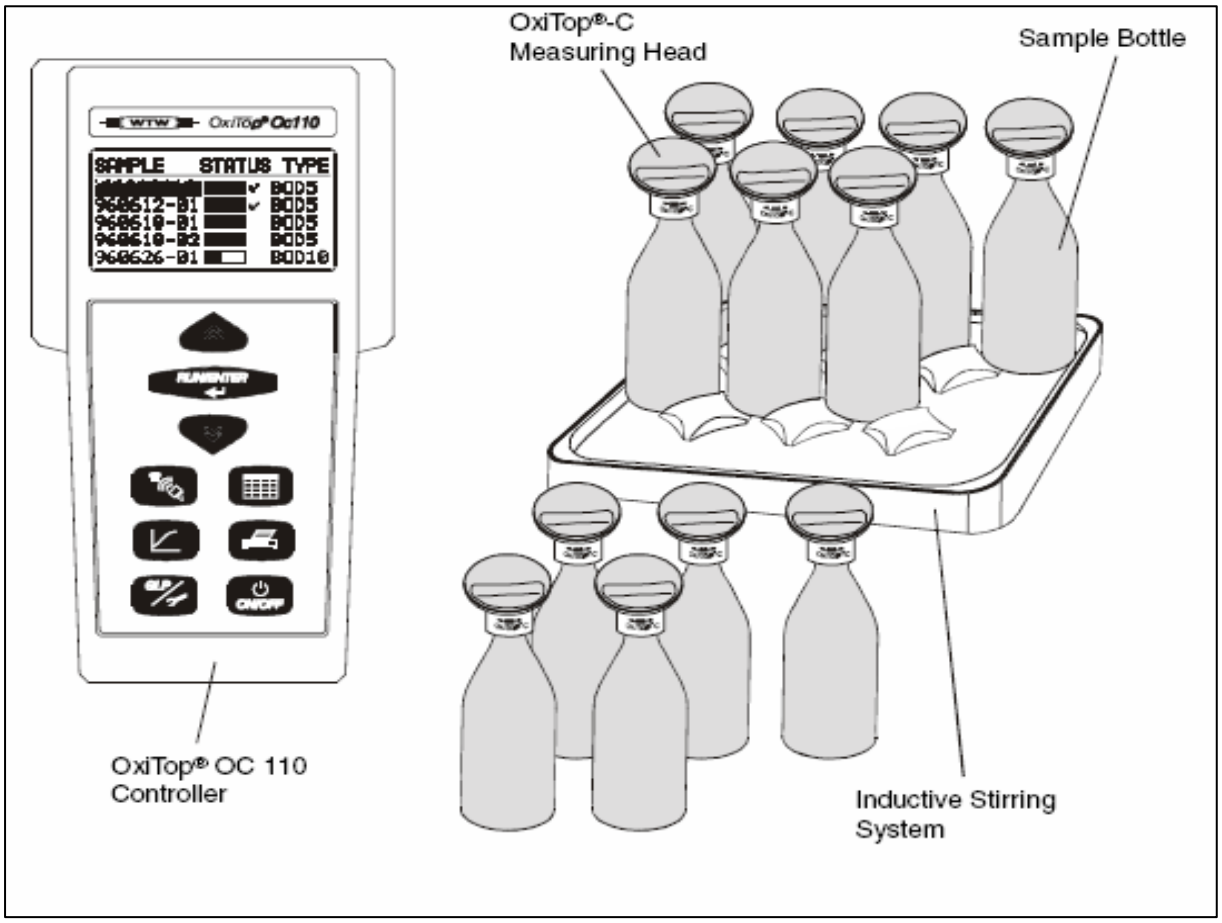


*Figure 1. Set-up aqueous biodegradation test*

## 7.3 Analytical methods

### Ammonium - nitrogen ($NH_4^+$-N)

This analysis is done as described in 'M_054. Determination of ammonium nitrogen by a discrete analyzer system and spectrophotometric detection'. The determination is performed on the aqueous sample after filtration through a syringe filter (pore size = 1.20 µm). Ammonia in the sample reacts with hypochlorite ions generated by the alkaline hydrolysis of sodium dichloroisocyanurate to form monochloramine. This reacts with salicylate ions in the presence of sodium nitroprusside at around pH 12.6 to form a blue compound. The absorbance of this compound is measured spectrophotometrically at the wavelength 660 nm and is related to the ammonia concentration by means of a calibration curve. The results are given in mg 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

The elemental analysis for calculation of the ThOD is performed by an external lab. CHN is measured according to DIN 51732 (2014) and 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 ($K_2SO_4$-Se-mixture) and under boiling conditions (380°C) with a mixture of sulphuric acid bound nitrogen is converted into the salt $(NH_4)_2SO_4$. 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_055. Determination of total oxidized nitrogen by a discrete analyzer system and spectrophotometric detection'. The determination is performed on the aqueous sample after filtration through a syringe filter (pore size = 1.20 µm). Nitrate in the sample is reduced to nitrite by hydrazine under alkaline conditions. The total nitrite ions are then reacted with sulphanilamide and N-1-napthylethylenediamine dihydrochloride under acidic conditions to form a pink azo-dye. The absorbance is measured at 540 nm and is related to the Total Oxidized Nitrogen ($NO_x^-$-N) concentration by means of a calibration curve. In order to measure only nitrite in the sample the reduction of nitrate by hydrazine is omitted. The concentration of nitrate can then be calculated by subtracting the concentration of nitrite from the concentration of total oxidized nitrogen. The results are given in mg 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 (2019), 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 $K_2CO_3$), 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 $K_2CO_3$ 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_017. Determination of total organic carbon - total carbon after dry combustion and inorganic carbon after acidification'. The total carbon content is determined using a high temperature (950°C to 1200°C) combustion method. The formed $CO_2$ is measured with infrared (IR) detection. Total inorganic carbon is measured by acidification of the sample and heating at 150°C. The sample is first incinerated in a muffle furnace at 550°C. The leftover ashes are subsequently acidified. The released $CO_2$ is determined by IR detection. The results are given in percent on wet weight.

## Total suspended solids (TSS) & volatile suspended solids (VSS)

This analysis is done as described in 'M_019. Determination of suspended solids by filtration through glass-fibre filter'. A known quantity of sample is filtered through a glass fibre filter using under pressure created by a vacuum pump. After filtration the filter is washed with water in order to remove soluble solids. The filter is then dried at 105°C and the amount of dry residue is weighed. Afterwards the filter is incinerated at 550°C and the amount of ash residue is weighed. The results are given in g/l.

## 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, several balances are used, with an accuracy of 0.1 mg for the determination of dry and volatile matter and weighing of the reference and test item, and an accuracy of 0.01 g for weighing of the mineral medium and inoculum.

# 8 Results

## 8.1 Test conditions and set-up

A set of 6 equal reactor vessels with a total volume of 500 ml each was used. Each reactor was filled with 250 g of test medium, consisting of 245 g of mineral medium and 5 g of inoculum. Reference item Sodium acetate was added as powder, while liquid test item CF-7 was added as such. The test set-up is given in Table 1. At start-up 25 mg of reference item Sodium acetate was added. Due to the low dry matter content, a higher amount of test item CF-7 was added in order to obtain a similar dose of reference and test item on dry weight basis. Also, allylthiourea was added to each reactor at start to prevent nitrification. 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 21°C ± 1°C in the dark. The total test duration was 28 days.

*Table 1. Test set-up aqueous biodegradation test*

| RN | Test series | Min. medium (g) | Inoculum (g) | Item (mg) |
|---|---|---|---|---|
| 1 | Control | 245 | 5 | - |
| 2 | Control | 245 | 5 | - |
| 3 | Sodium acetate | 245 | 5 | 24.9 |
| 4 | Sodium acetate | 245 | 5 | 25.1 |
| 5 | CF-7 | 245 | 5 | 352.1 |
| 6 | CF-7 | 245 | 5 | 350.1 |

*RN = reactor number*

## 8.2 Analyses of inoculum, reference and test item

The inoculum consisted of a mixture of activated sludge from different sewage-treatment plants (Destelbergen, Landegem and Gent) treating domestic and/or industrial wastewater. After filtration over an 80 µm sieve, mixing in equal parts, decantation of the supernatant and replacement with mineral medium, the final sludge inoculum was obtained. This inoculum was actively aerated for 1 hour. The final test medium is obtained by adding 5 g of sludge inoculum to 245 g of mineral medium. As a result, a total suspended solids content of 40.9 mg/l and a volatile suspended solids content of 33.4 mg/l were obtained in the final test medium. The total suspended solids (TSS), volatile suspended solids (VSS) and Kjeldahl nitrogen of the sludge inoculum itself are given in Table 2, together with the pH and nitrogen analyses of the test medium (= sludge inoculum + mineral medium, but without test item).

*Table 2. Characteristics of inoculum and test medium*

| Characteristics | Result |
|---|---|
| **Inoculum** | |
| Total suspended solids (TSS, g/l) | 2.04 |
| Volatile suspended solids (VSS, g/l) | 1.67 |
| Volatile suspended solids (VSS, % on TSS) | 82.3 |
| Kjeldahl-N (mg/l) | 20 |
| **Test medium** | |
| pH | 7.1 |
| $NH_4^+$-N (mg/l) | 0.5 |
| $NO_x^-$-N (mg/l) | 0.4 |

The total solids (TS), volatile solids (VS), theoretical oxygen demand (ThOD, calculated from elemental analysis), total organic carbon content (TOC) and theoretical amount of evolved carbon dioxide ($ThCO_2$) of the reference and test item are summarised in Table 3.

*Table 3. TS, VS, ThOD, TOC and $ThCO_2$ of reference and test item*

| Item | TS (%) | VS (% on TS) | ThOD (mg/g) | TOC (%) | $ThCO_2$ (mg/g) |
|---|---|---|---|---|---|
| Sodium acetate | 95.4 | 35.3 | 744 | 28.8 | 1056 |
| CF-7 | 7.0 | 98.5 | 69 | 3.0 | 110 |

## 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 reference or test 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. No nitrate or nitrite was detected, so no correction for $O_2$ consumption due to nitrification needed to be made.

Table 4 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.

According to OECD 301F (1992) the oxygen consumption of the controls is normally 20 - 30 mg $O_2$/l and should not be greater than 60 mg $O_2$/l in 28 days. The total $O_2$ consumption of the controls after 28 days of testing was 17.0 ± 2.8 mg $O_2$/l medium. The requirement was fulfilled.

*Table 4. ThOD, net $O_2$ consumption and biodegradation after 28 days*

| Test series | ThOD (mg/l) | Net $O_2$ (mg/l) | Biodegradation (%) | | |
|---|---|---|---|---|---|
| | | | AVG | SD | REL |
| Sodium acetate | 74.4 | 69.1 | 92.8 | 3.3 | 100.0 |
| CF-7 | 97.1 | 97.0 | 99.8 | 2.4 | 107.5 |

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

The biodegradation of the reference item Sodium acetate started at a good rate. After 4 days Sodium acetate was already degraded by 64.7%. The biodegradation rate gradually decreased and after 28 days (end of test) a plateau in biodegradation was reached at a level of 92.8% ± 3.3%. The test is considered valid if after 14 days the biodegradation percentage of the reference item is more than 60%. This requirement was fulfilled.

The biodegradation of test item CF-7 started after approximately one day and proceeded at a good rate. After 9 days the test material was already degraded by 62.9%. As such the 60% biodegradability requirement has been reached within 10 days. The biodegradation rate gradually slowed down and at the end of the test (28 days) a biodegradation of 99.8% ± 2.4% was measured.

The test is considered valid if the difference in biodegradation between the test item replicates is less than 20% at the end of the test. After 28 days (end of test) a difference of 3.4% was calculated for test item CF-7. The requirement was fulfilled.

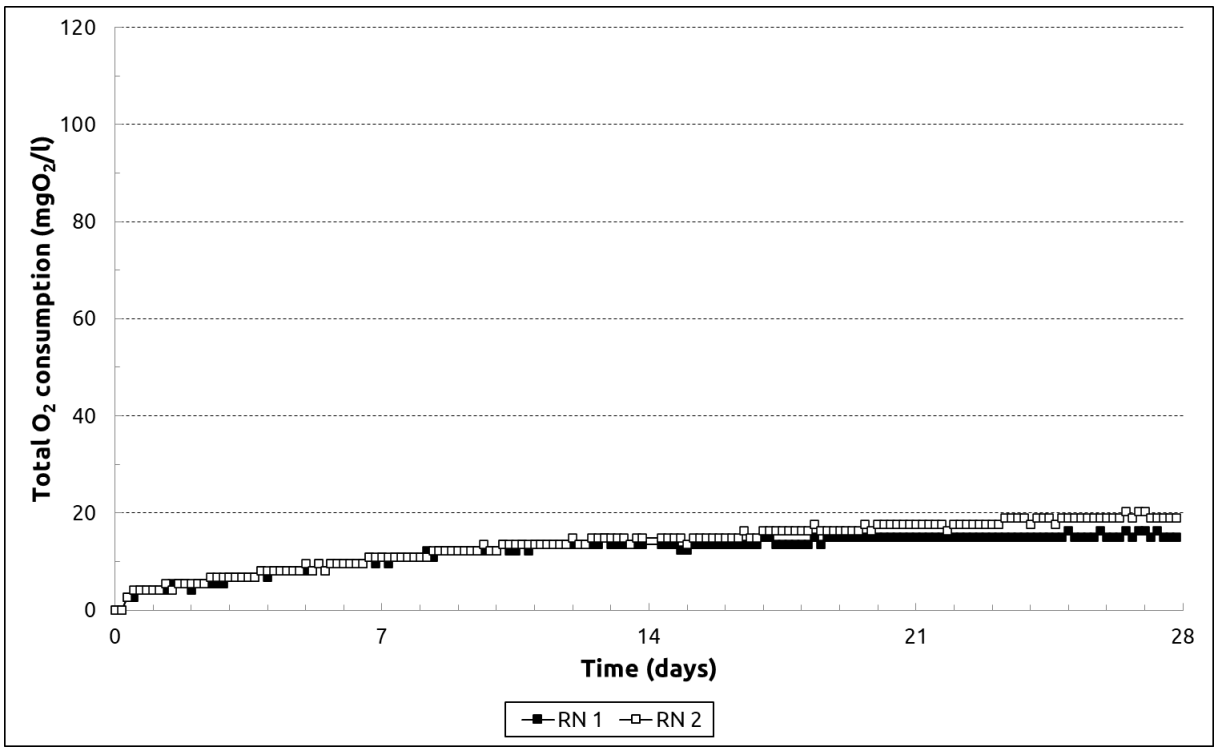


*Figure 2. Total $O_2$ consumption of control reactors*

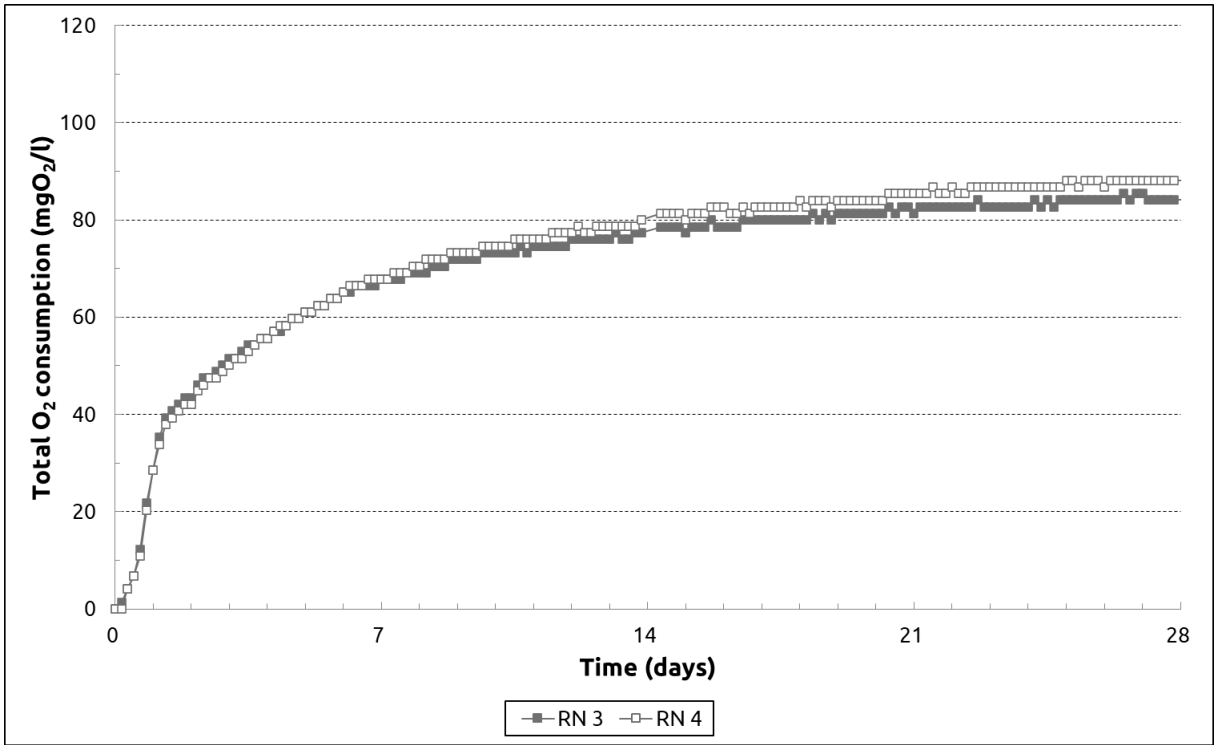


*Figure 3. Total $O_2$ consumption of Sodium acetate reactors*

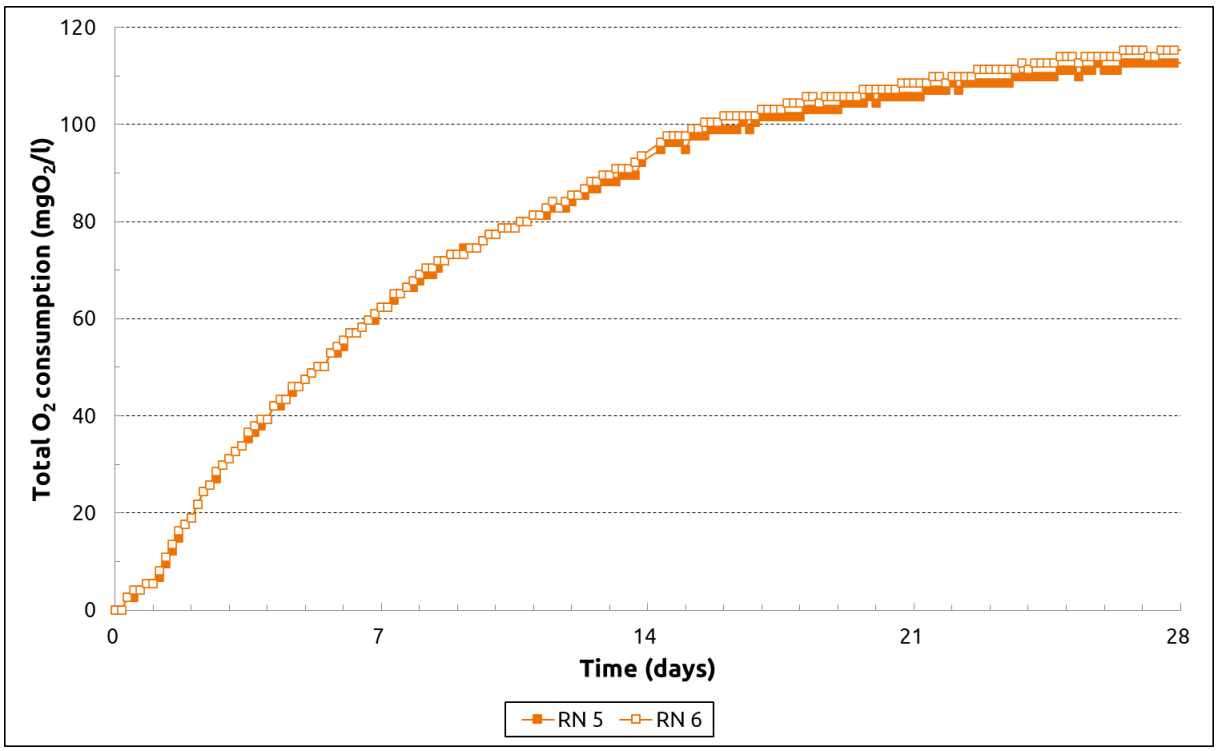


*Figure 4. Total $O_2$ consumption of CF-7 reactors*

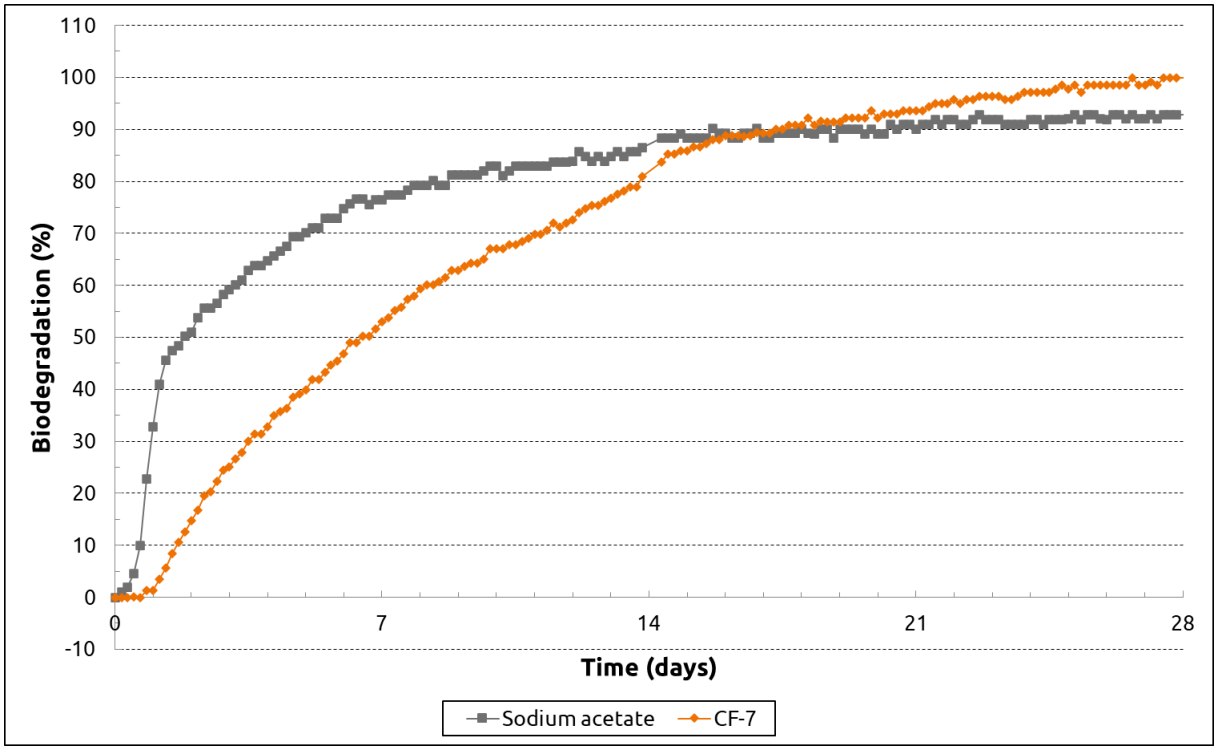


*Figure 5. Evolution of the average biodegradation percentage of reference and test item (based on $O_2$ consumption)*

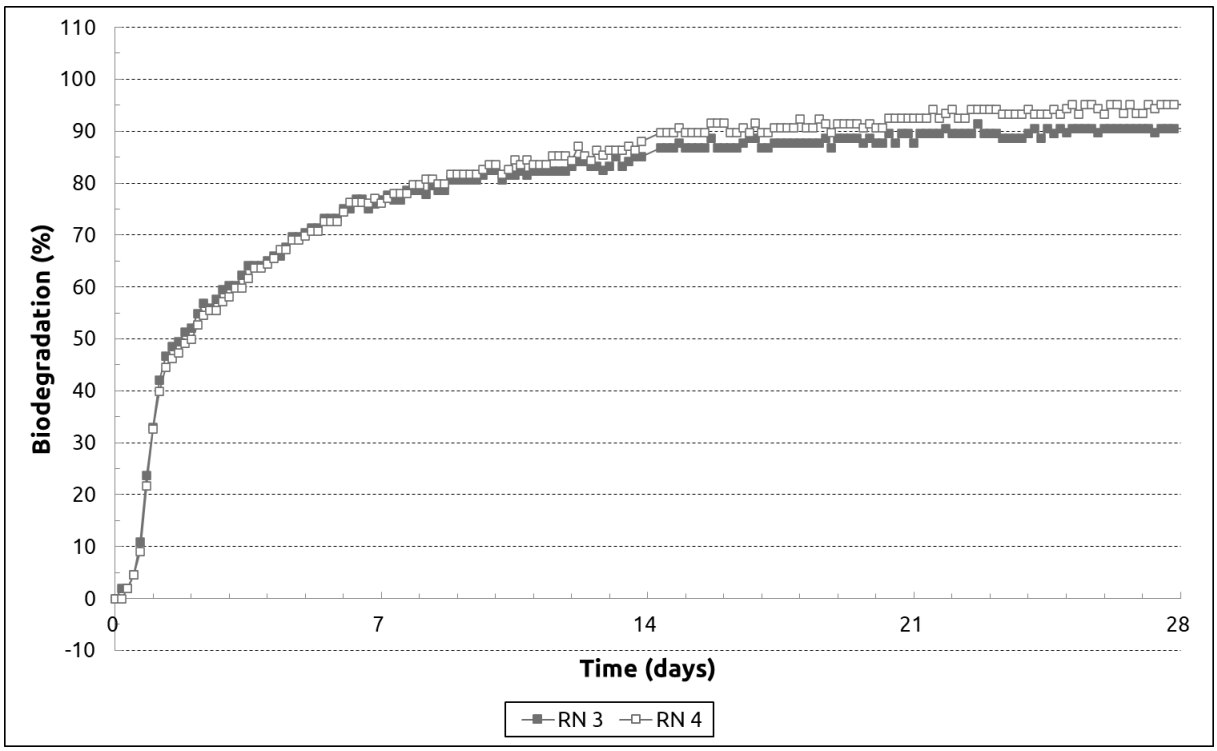


*Figure 6. Evolution of the biodegradation percentage of replicates of Sodium acetate (based on $O_2$ consumption)*

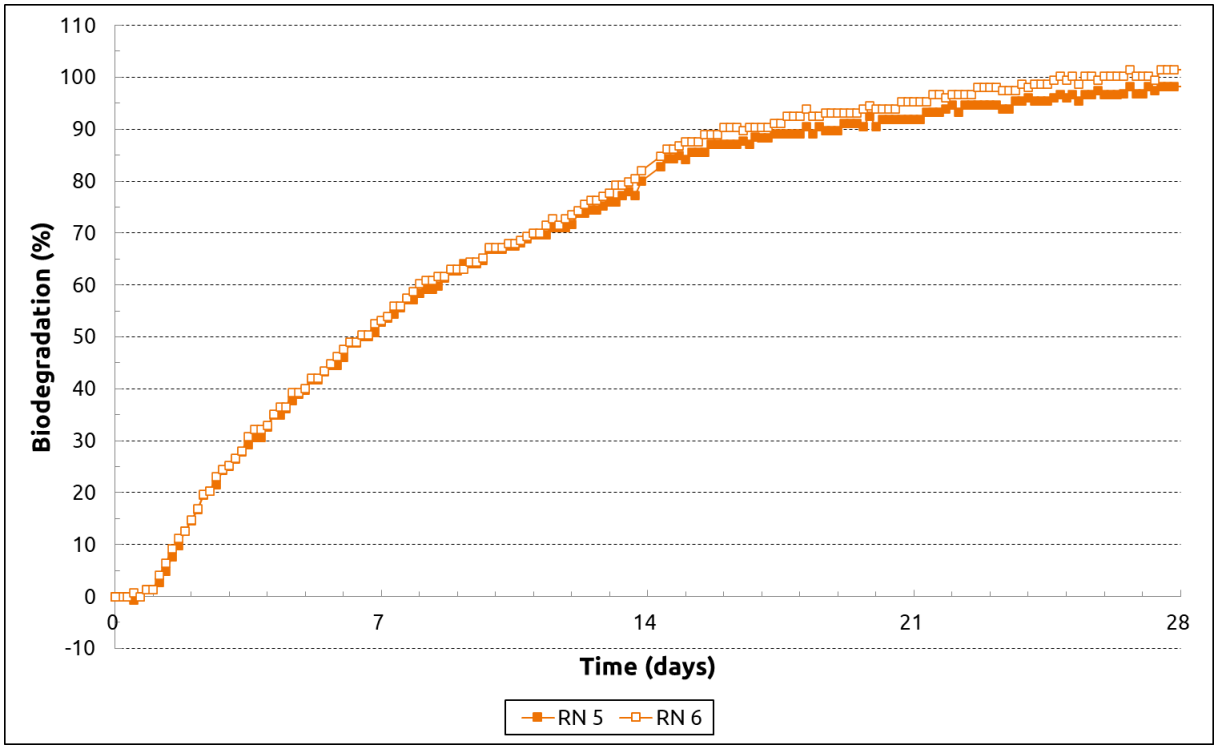


*Figure 7. Evolution of the biodegradation percentage of replicates of CF-7 (based on $O_2$ consumption)*

### 8.3.2 Biodegradation based on $CO_2$ production

The biodegradation was also determined by measuring the amount of $CO_2$ that had been captured in the KOH solution during the test.

Table 5 shows the $ThCO_2$ (= theoretical $CO_2$ production based on the % organic C and input of the item), net $CO_2$ production and biodegradation percentage of reference and test item at the end of the test (28 days). A visual presentation of the cumulative $CO_2$ 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 $CO_2$ production), while Figures 12 and 13 show the biodegradation of the replicates.

*Table 5. $ThCO_2$, net $CO_2$ production and biodegradation after 28 days*

| Test series | $ThCO_2$ (mg) | Net $CO_2$ (mg) | Biodegradation (%) | | | 95% CL |
|---|---|---|---|---|---|---|
| | | | AVG | SD | REL | |
| Sodium acetate | 26.4 | 22.0 | 83.4 | 1.2 | 100.0 | 23.9 |
| CF-7 | 38.6 | 30.9 | 80.0 | 2.3 | 96.0 | 16.7 |

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

The test is considered valid if after 14 days the biodegradation percentage of the reference item Sodium acetate is more than 60%. After 14 days an absolute biodegradation of 77.3% was measured. At the end of the test (28 days) a plateau in biodegradation was reached at a level of 83.4% ± 1.2%. The requirement was fulfilled.

The biodegradation of test item CF-7 proceeded at a good rate throughout the test. After 14 days the test material was degraded by 64.6% and at the end of the test (28 days) an absolute biodegradation of 80.0% ± 2.3% was measured. On a relative basis, compared to suitable reference substrate Sodium acetate, a biodegradation of 96.0% was calculated.

The test is considered valid if the difference in biodegradation between the test item replicates is less than 20% at the end of the test. After 28 days (end of test) a difference of 3.2% was calculated for test item CF-7. The requirement was fulfilled.

A test item is considered to meet the biodegradation requirement of OECD *Guideline for Testing of Chemicals 301F – Manometric Respirometry Test* (1992) if 60% removal of ThOD or $ThCO_2$ is achieved. The pass level must be reached in a 10-day window within the 28-day period of the test for a chemical to be considered readily biodegradable. The 10-day window begins when the degree of biodegradation has reached 10% ThOD or $ThCO_2$ and must end before day 28 of the test. From these results it can be concluded that test item CF-7 fulfilled the 60% biodegradability requirement within 28 days of testing under the given aerobic conditions. Moreover, as the 60% pass level was reached with a 10-day window, test item CF-7 can be considered readily biodegradable.

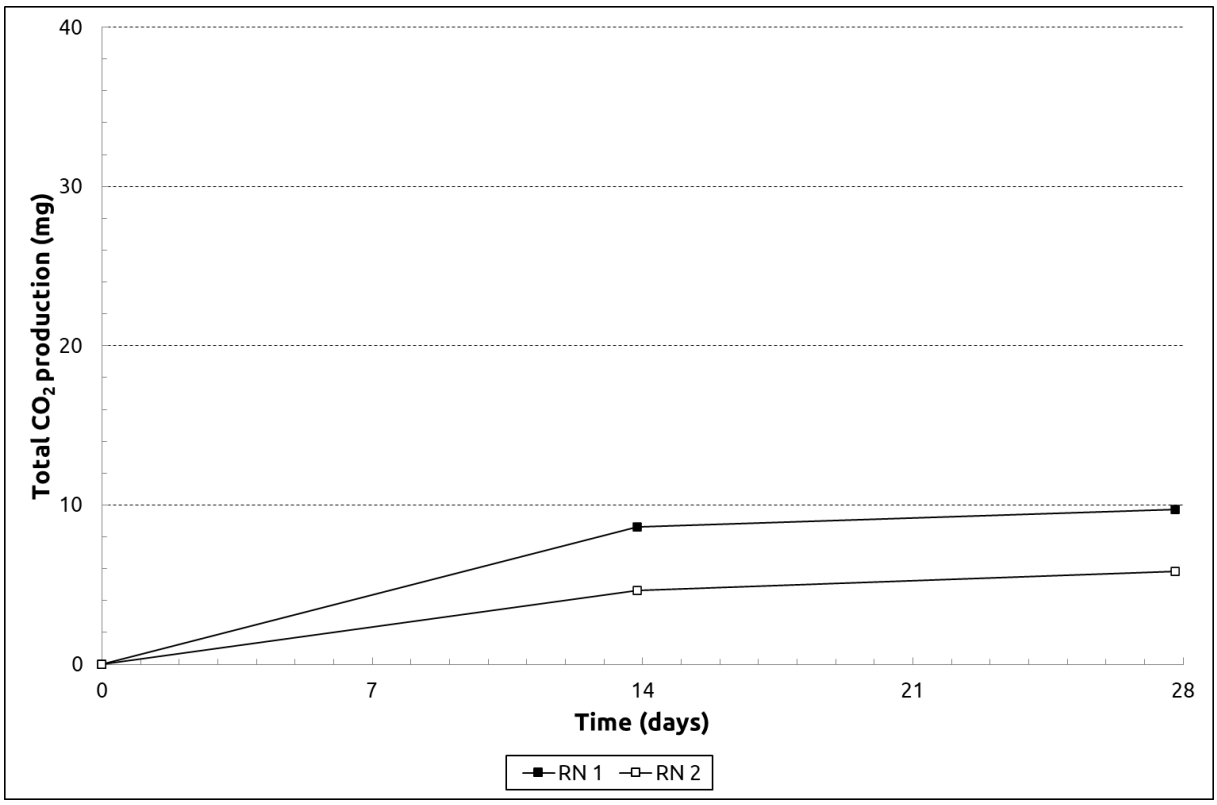


*Figure 8. Total $CO_2$ production of control reactors*

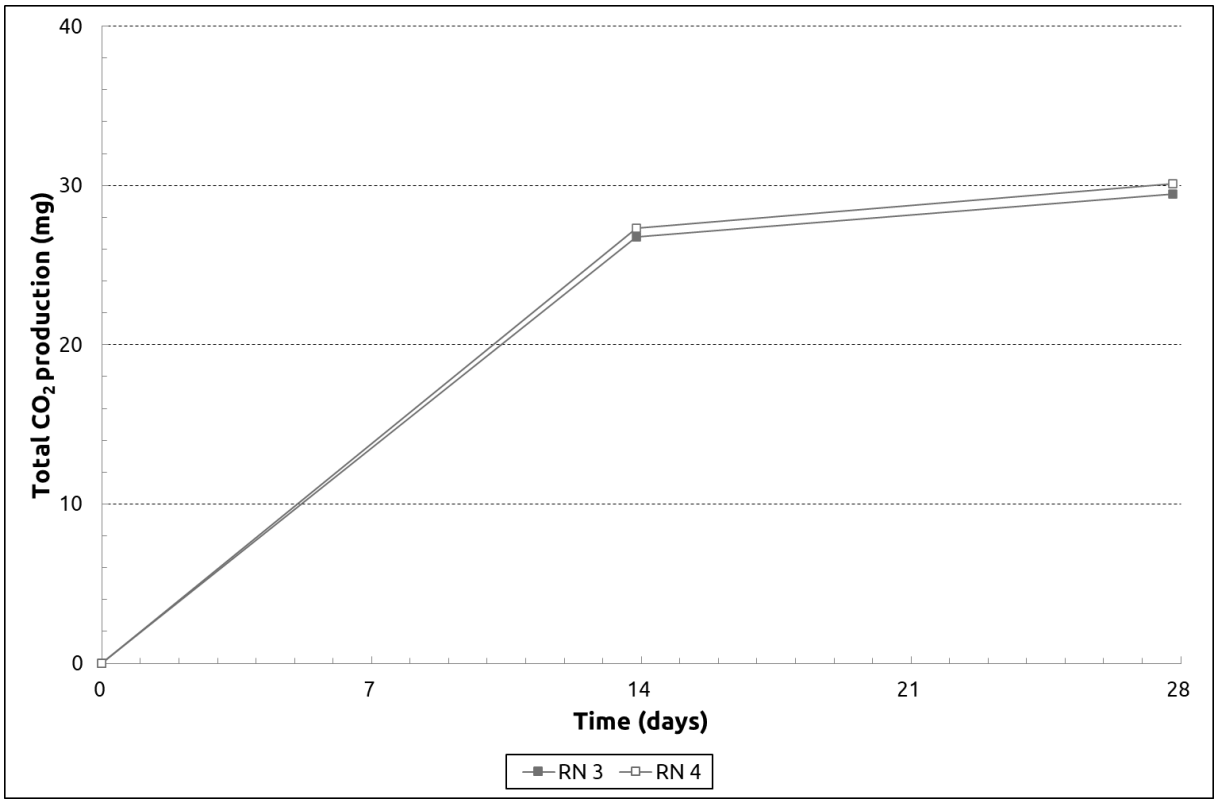


*Figure 9. Total $CO_2$ production of Sodium acetate reactors*

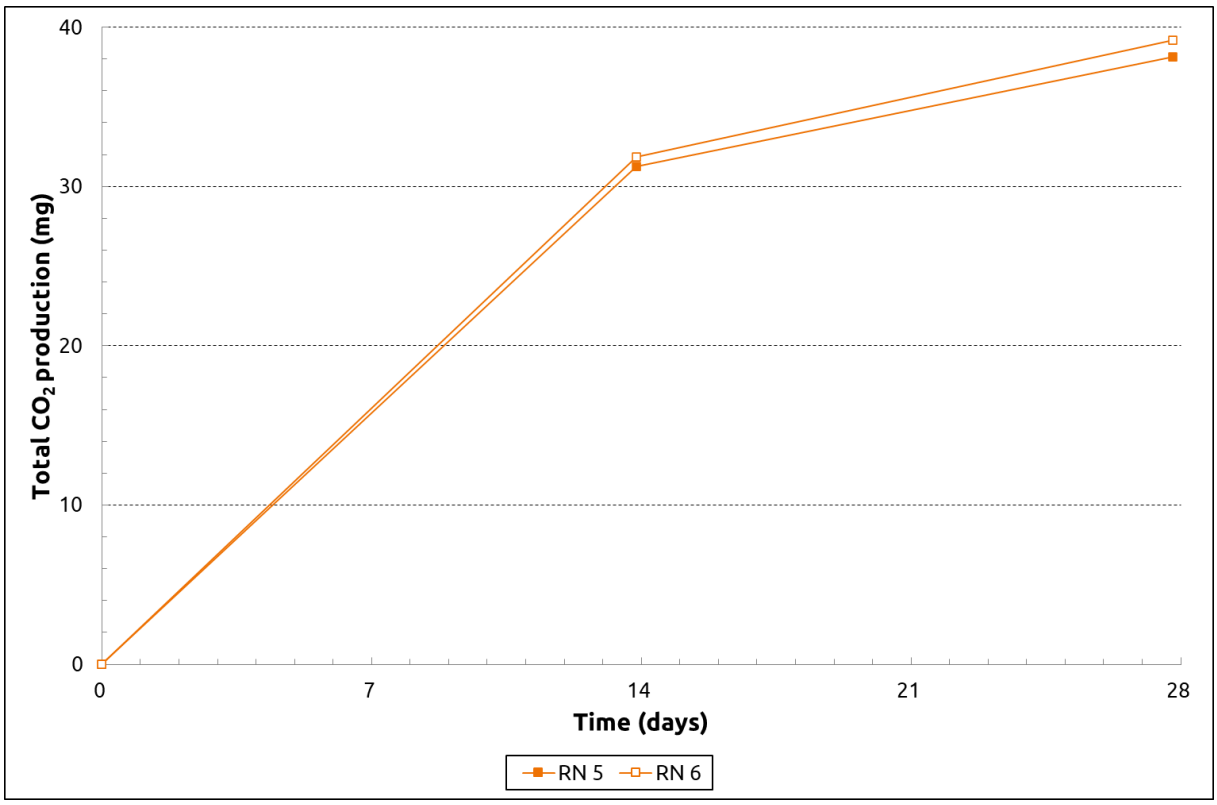


*Figure 10. Total $CO_2$ production of CF-7 reactors*

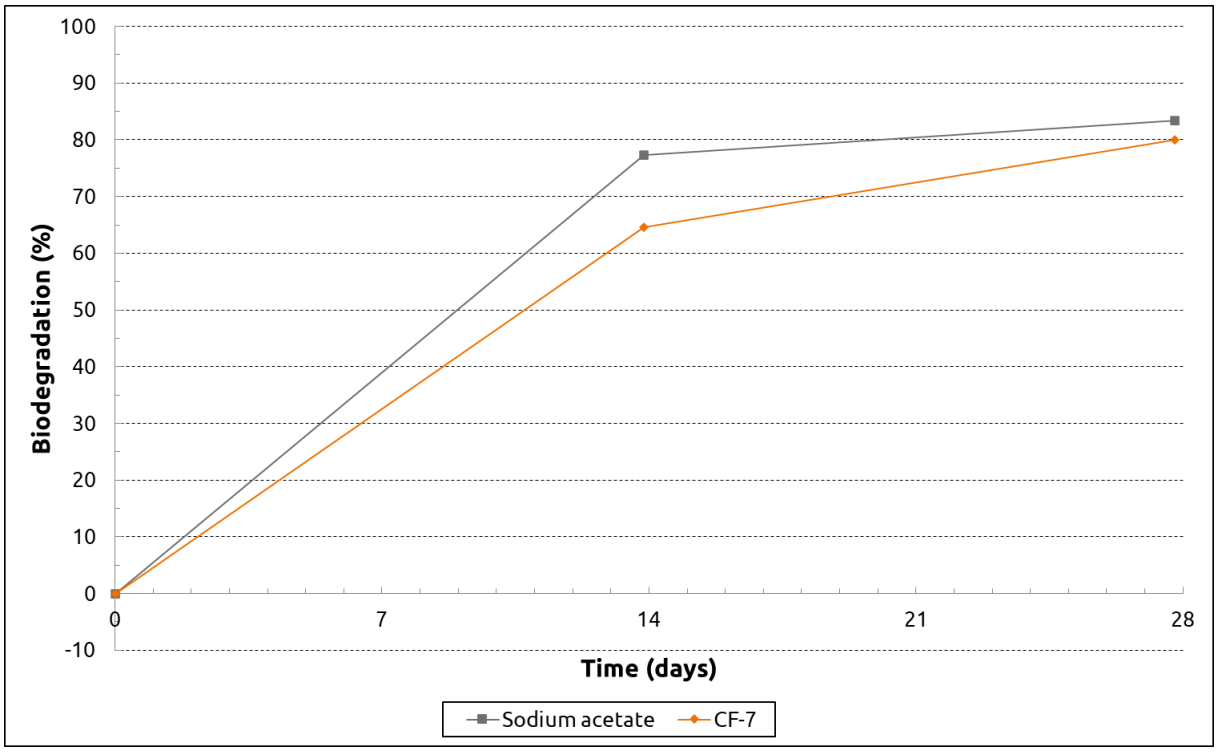


*Figure 11. Evolution of the average biodegradation percentage of reference and test item (based on $CO_2$ production)*

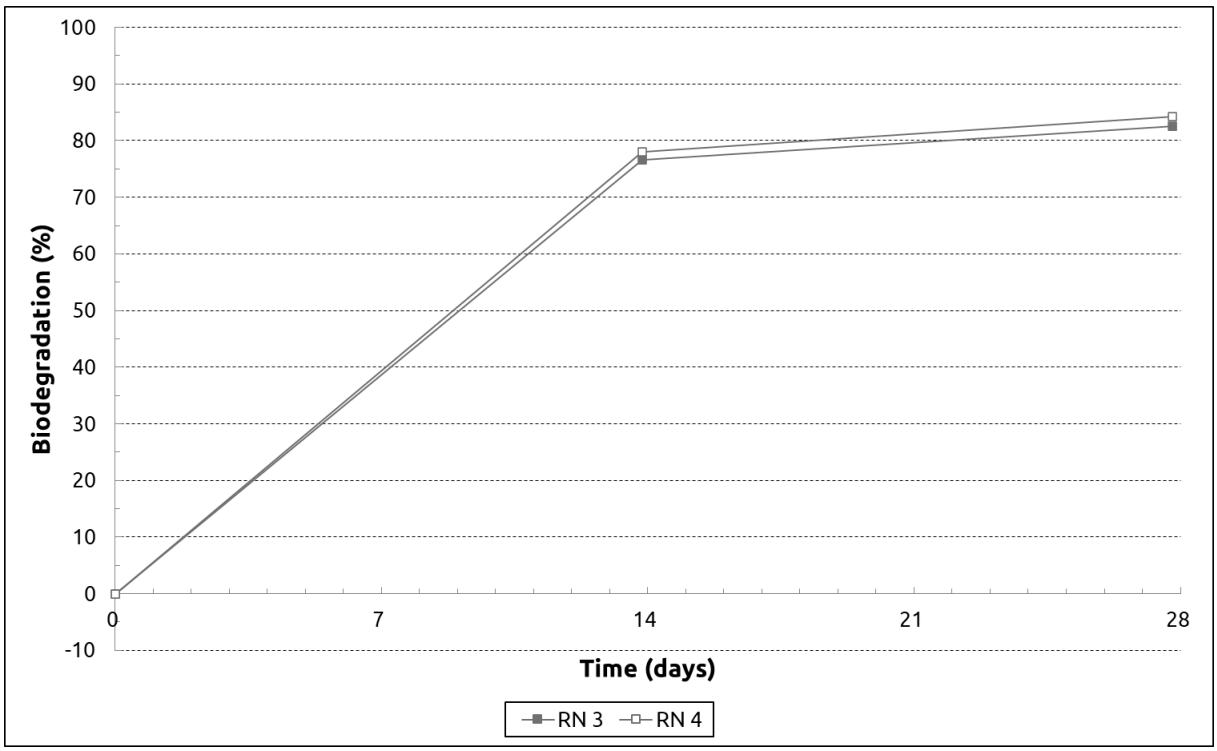


*Figure 12. Evolution of the biodegradation percentage of replicates of Sodium acetate (based on $CO_2$ production)*

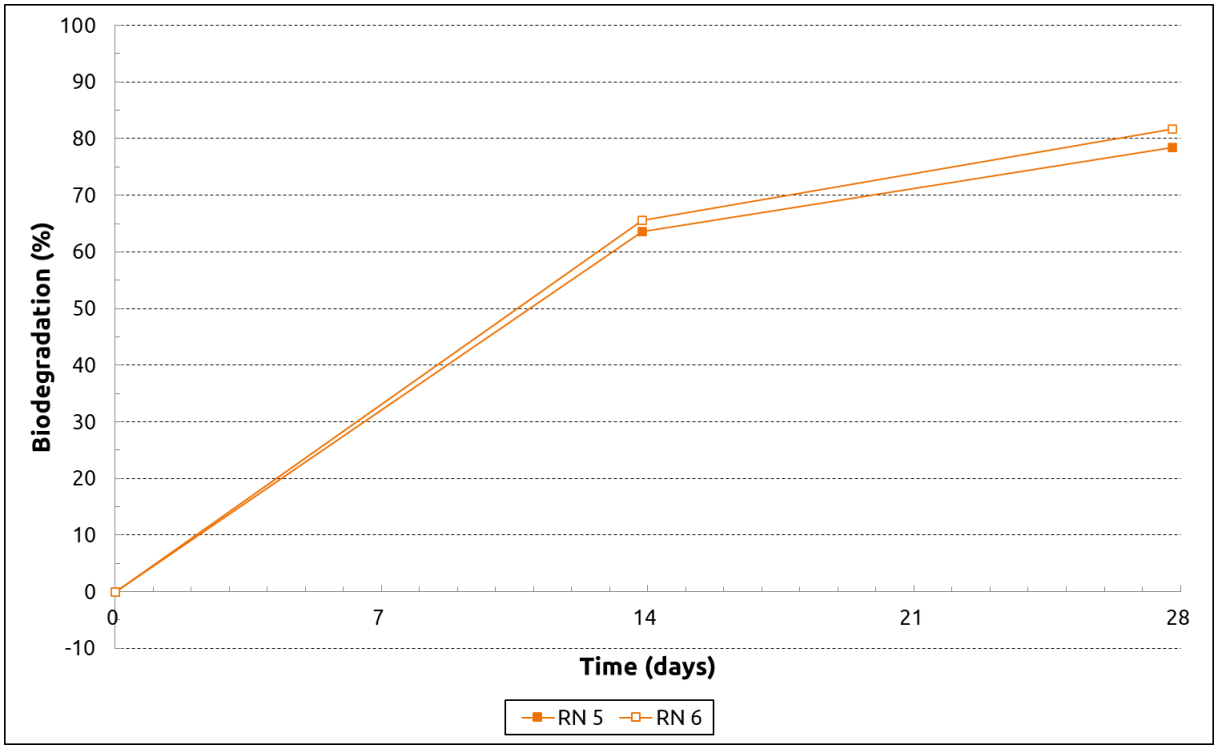


*Figure 13. Evolution of the biodegradation percentage of replicates of CF-7 (based on $CO_2$ production)*