

# **FINAL REPORT OF *IN VIVO* TEST**

## **SUMMARY**

**Date:** January 9<sup>th</sup>, 2013

**Report No.** AP01/13

**Applicant Company:** Special Nutrients, Inc.

**Applicant:** Fernando Tamames

**Address:** 2766 SW Douglas Rd, Miami, Florida 33133 – USA

**Animal Species Tested:** Fish

**Micotoxins Tested:** Aflatoxins

**Start of the Experiment:** December 12<sup>th</sup>, 2012

**End of the Experiment:** January 2<sup>nd</sup>, 2013

**Parameters Evaluated:** Data of Average Weight and Average Total Size of animals.

Study performed in conformity with the recommendations of the Task Force of the Ministry of Agriculture, Livestock and Provision (MAPA) for the register of Anti-Micotoxin Additives. Experiment performed in conformity with Resolution No. 879 of February 15<sup>th</sup>, 2008 for animal testing and animal wellbeing.

**Result:** This report presents an Anti-Micotoxin Additive (AMA) that complies with the minimum requirements for approval.

## **Efficacy of the Anti-Micotoxin Additive TOXFREE STANDARD in the reduction of the toxic effects of Aflatoxin added to fish diets.**

This study was carried out upon request of the company SPECIAL NUTRIENTS INC., with the objective to evaluate the efficacy of the Anti-Micotoxin Additive (AMA) TOXFREE STANDARD in the reduction of toxic effects of aflatoxins added to fish diets.

The experiment was performed by Instituto SAMITEC (Institute of Analytical, Microbiological and Technological Solutions) during the period from Dec 12<sup>th</sup>, 2012 and Jan 2<sup>nd</sup>, 2013.

### **FACILITIES**

The study was performed in an experimental unit of SAMITEC. The experimental room, measuring 22 m<sup>2</sup>, was maintained at an ideal temperature for the development of the animals of the age group used (25°C). Negative pressure was maintained throughout the experiment. Fishes were allocated in 24 polypropylene cages with an individual capacity of 60 L each, for a total of 2500 L of a recirculating water system. Water recirculation was maintained at 3 L per minute, for 24h, with two biofilters built into the system.

Water temperature was controlled by an electric heater regulated by a thermostat. The daily water renovation rate was approximately 5%, considering the need for clearance of dejections and feed waste. Physic-chemical analysis of water was performed using a kit (Alfa Tecnoquimica) measuring ammonia nitrogen, dissolved oxygen, pH, water transparency, total alkalinity, total water hardness and temperature (Annex Figure 3).

### **ANIMALS AND FEEDING**

Two hundred and eighty eight, 288, fishes from the species *Oreochromis niloticus*, known as Tilapia of the Nile were used for the experiment. Average weight and total size at the start of the experiment (day 0) were 2.64 g and 3.2 cm, respectively. Handling of fishes was the same as used routinely for fish housed in cages: fishes were fed twice a day (8 and 18 hours), an amount equivalent to 5% of total biomass. Amount of diet fed was adjusted on days 0, 7 and 14 of the study, based on a sample of 100% of fishes included in the experiment. On weighing days, animals were fasted 6h before the measurement. The diet was isonutritious for the total duration of the experiment and its composition is represented on Table 1.

**Table 1. Nutritional Levels of Fish Diets**

<b>Nutrients</b>	<b>Initial Diet (1-21days)</b>
Crude Protein (%)	35.22
Metabolizable Energy (kcal/kg)	3,440.40
Calcium (%)	3.20
Available Phosphorus (%)	1.87
Crude Fiber (%)	2.54
Dry Matter (%)	90.73
Ashes (%)	10.65
Ether Extract (%)	16.16

## EXPERIMENTAL DESIGN

A Completely Randomized design was used, with the 288 fishes distributed in 5 treatments, with 4 repetitions for treatment 1 and 5 repetitions for treatments 2, 3, 4 and 5, with 12 fishes in each repetition. Treatments were established as shown on Table 2.

**Table 2. Level of Micotoxin and TOXFREE STANDARD added to diet**

Treatment	Number of fish	Aflatoxin (ppm)	TOXFREE STANDARD (%)
1	48	-	-
2	60	-	0.50
3	60	5.0	-
4	60	5.0	0.25
5	60	5.0	0.50

## MICOTOXINS USED IN THE STUDY

Aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) were obtained from a culture of a toxigenic strain of *Aspergillus parasiticus*. Concentration of aflatoxins used in the study are shown in Table 3.

**Table 3. Type and Concentration of Aflatoxins used**

Aflatoxin Type	Concentration (%)
B <sub>1</sub>	93.80
B <sub>2</sub>	2.10
G <sub>1</sub>	3.40
G <sub>2</sub>	0.70

## EXPERIMENTAL PARAMETERS

The following parameters were measured weekly (0, 7, 14 and 21 days of age)

- Live Weight of fishes obtained by individual measurement.
- Fish Size: Measured according to NAFO (Northwest Atlantic Fisheries Organization). It was measured in a straight line from the cranial end of the face to the end of the longest lobe of the tail fin when both lobes are depressed on the midline.

## STATISTICAL ANALYSIS

The statistical analysis was performed using descriptive statistical analysis (mean and coefficient of variation). Analysis of Variance was performed using Bonferroni test ( $P \leq 0.05$ ) for comparison of means. The analysis were done using Stagraphics Centurion XV version 15.1 software package.

## RESULTS AND DISCUSSION

### AVERAGE WEIGHT OF FISHES

Table 4 and Figure 1 show data on average weight of fishes from the start to d7, 14 and 21 of the experiment.

**Table 4. Average Weight (g) of fishes fed a diet containing aflatoxin, with and without the addition of TOXFREE STANDARD, during 21 days.**

Treatment	d0		d7		d14		d21	
	ABW	CV	ABW	CV	ABW	CV	ABW	CV
Control	2.74 <sup>a</sup>	21.0	5.02 <sup>a</sup>	21.9	7.61 <sup>a</sup>	22.3	11.17 <sup>a</sup>	19.1
0.50% Toxfree Std	2.73 <sup>a</sup>	20.0	4.85 <sup>a</sup>	22.1	7.55 <sup>a</sup>	22.2	10.66 <sup>ab</sup>	21.1
5 ppm Aflatoxin	2.65 <sup>a</sup>	27.8	4.45 <sup>a</sup>	27.7	6.02 <sup>b</sup>	29.9	8.24 <sup>d</sup>	26.3
5ppm Afla + 0.25% Tx Std	2.48 <sup>a</sup>	30.6	4.45 <sup>a</sup>	29.7	6.18 <sup>b</sup>	30.9	8.40 <sup>cd</sup>	28.4
5ppm Afla + 0.50% Tx Std	2.58 <sup>a</sup>	25.1	4.59 <sup>a</sup>	22.6	6.76 <sup>ab</sup>	23.3	9.52 <sup>bc</sup>	22.8
Mean	2.64	24.9	4.67	24.8	6.83	25.7	9.60	23.5

<sup>a-d</sup> Means in the same column with different superscripts differ by Bonferroni test ( $P \leq 0.05$ )

ABW = Average Body Weight (g)

CV = Coefficient of Variance (%)

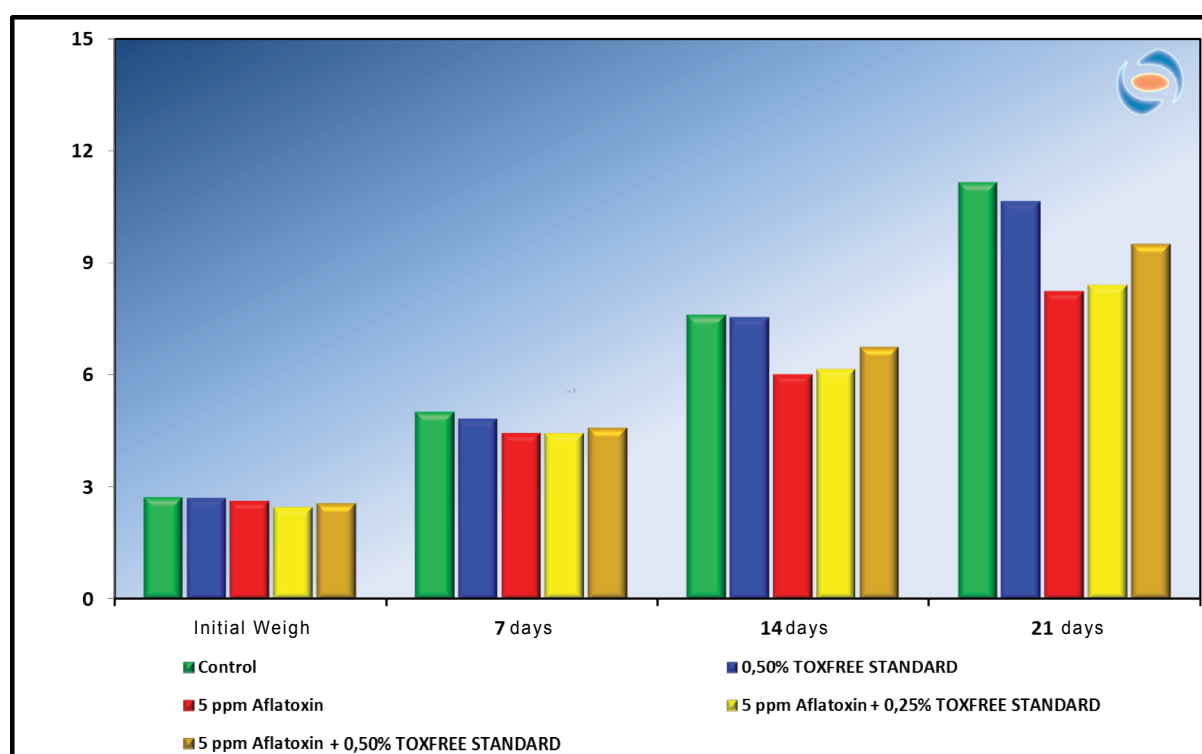


Figure 1. Average Weight (g) of fishes fed a diet containing aflatoxins, with and without the addition of TOXFREE STANDAR during 21 days.



## Discussion

Table 4 and Figure 1 show the average weight of fishes at time of allocating (2.64 g). The weight, associated to the coefficient of variation, is not different ( $P \leq 0.05$ ) across treatments, this being an important parameter in experiments evaluating micotoxicosis in fishes.

From the second week of the study, until the end, it can be observed the detrimental effect of aflatoxins on the weight of the animals. In this period, the presence of aflatoxins in the diet resulted in a lower body weight gain of fishes (- 26.2%) when comparing with those not ingesting aflatoxins. At the end of the experiment, fishes receiving aflatoxins and **TOXFREE STANDARD** (0.5%) showed a higher average body weight (+15.5%) than fishes receiving aflatoxins without AMA in the diet.

## AVERAGE TOTAL SIZE OF FISHES

Table 5 and Figure 2 show the values for average total size of fishes from the start to d7, d14 and d21 of the study.

**Table 5. Average Total Size (cm) of fishes fed a diet containing aflatoxin, with and without the addition of TOXFREE STANDARD, during 21 days.**

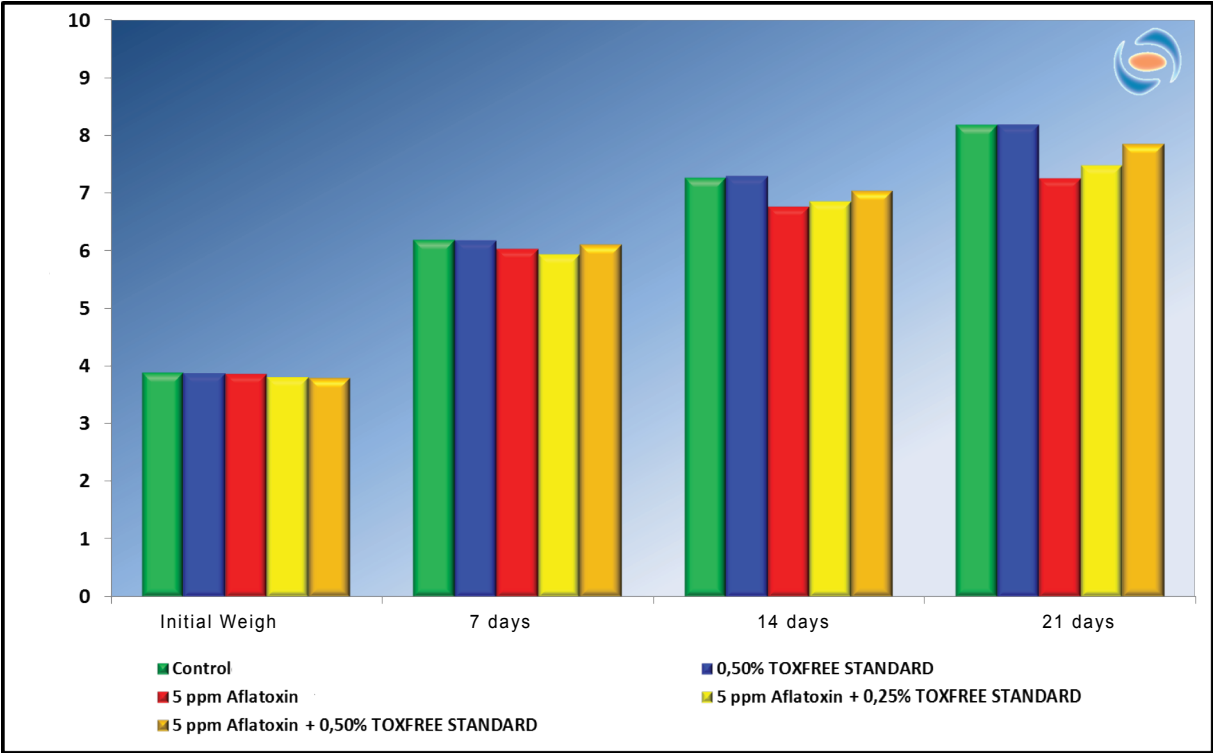
Treatment	d0		d7		d14		d21	
	ATS	CV	ATS	CV	ATS	CV	ATS	CV
Control	3.89 <sup>a</sup>	7.6	6.19 <sup>a</sup>	7.6	7.27 <sup>a</sup>	8.6	8.19 <sup>a</sup>	8.1
0.50% Toxfree Std	3.89 <sup>a</sup>	6.6	6.19 <sup>a</sup>	8.0	7.30 <sup>a</sup>	9.1	8.19 <sup>a</sup>	8.6
5 ppm Aflatoxin	3.86 <sup>a</sup>	7.9	6.04 <sup>a</sup>	9.5	6.77 <sup>b</sup>	10.4	7.27 <sup>b</sup>	9.4
5ppm Afla + 0.25% Tx Std	3.82 <sup>a</sup>	9.3	5.95 <sup>a</sup>	10.2	6.86 <sup>b</sup>	9.6	7.49 <sup>b</sup>	8.5
5ppm Afla + 0.50% Tx Std	3.81 <sup>a</sup>	7.8	6.11 <sup>a</sup>	8.0	7.05 <sup>ab</sup>	8.6	7.87 <sup>a</sup>	6.5
Mean	3.85	7.8	6.10	8.6	7.05	9.2	7.80	8.23

<sup>a-d</sup> Means in the same column with different superscripts differ by Bonferroni test ( $P \leq 0.05$ )

ATS = Average Total Size (cm)

CV = Coefficient of Variance (%)

**Figure 2. Average Total Size (cm) of fishes fed a diet containing aflatoxin, with and without the addition of TOXFREE STANDARD, during 21 days.**



## **Discussion**

The effect of aflatoxins was significant from the second week of the experiment (d14) and it was maintained until the end (d21). The average total size of fishes receiving aflatoxins in the diet was lower (-11.2%) than the average total size of fishes from the control groups. The average total size of fishes receiving **TOXFREE STANDARD** (0.5%) in the diet was higher (+8.2%) than those with diets containing only aflatoxins.

## **CONCLUSIONS**

1. The harmful effects of the inclusion of aflatoxins at 5ppm concentration in the diet of fishes are clearly shown during a period of 21 days.
2. The parameters evaluated in this study showed a significant efficacy of the addition of the **AMA TOXFREE STANDARD** at an inclusion rate of 0.5% in fishes against a challenge of 5 ppm of aflatoxins during 21 days.

The results obtained in this experiment refer to the product provided by SPECIAL NUTRIENTS INC, and are specific for the sample provided. Any future modification in the product, regarding chemical or physical characteristics should result on a re-evaluation.

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Santa Maria, January 9<sup>th</sup>, 2013

## ANNEX



Figure 3. Picture of the facility used in the study

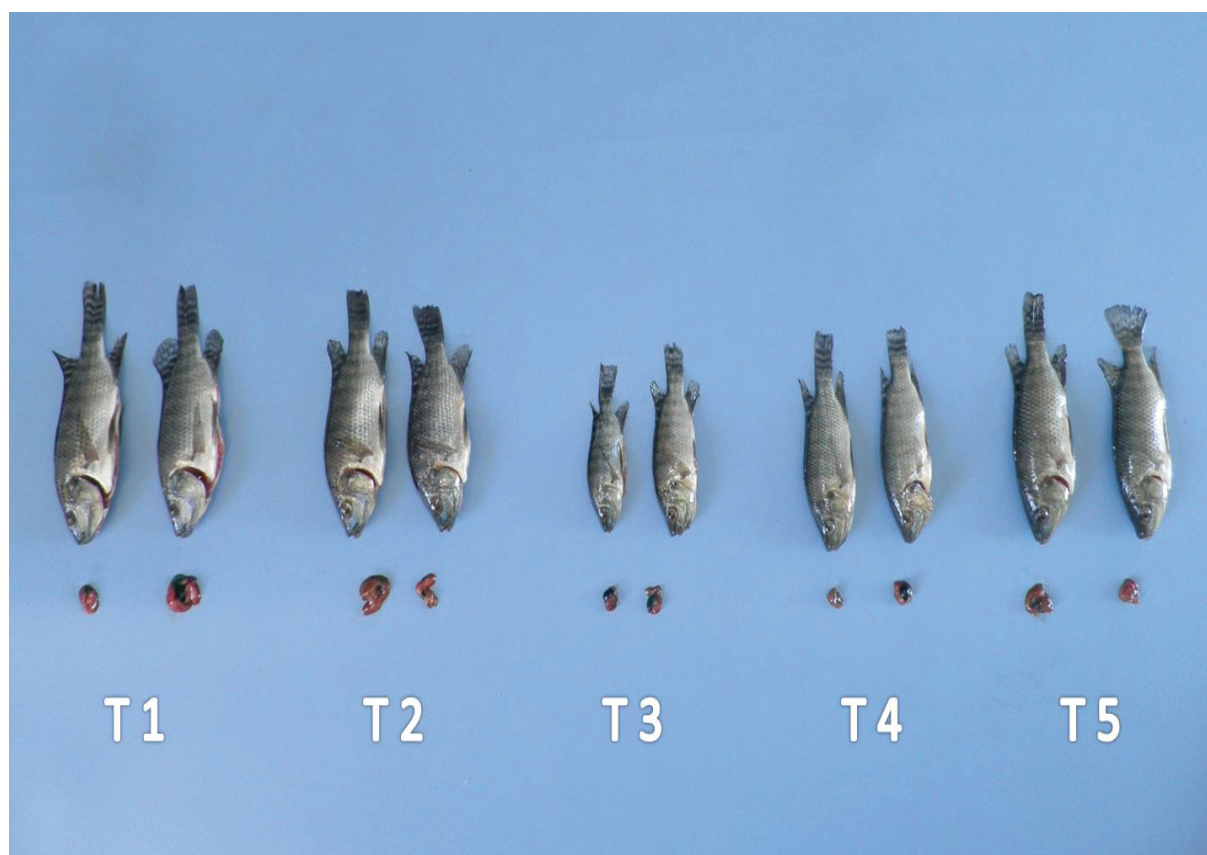


Figure 4. Picture of the body and liver development of fishes (T1 = Control; T2 = 0.50% TOXFREE STANDARD; T3 = 5ppm Aflatoxins; T4 = 5ppm Aflatoxins + 0.25% TOXFREE STANDARD; T5 = 5ppm Aflatoxins + 0.5% TOXFREE STANDARD).