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Control of Stored Agro-Commodity Pests *Sitophilus granarius* and *Callosobruchus chinensis* by Nitrogen Hypoxic Atmospheres: Laboratory and Field Validations

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Abstract: Given the complexity of the practical usage of controlled atmospheres for the protection of agro-commodities, several researchers have pointed out that there is not enough robust scientific documentation regarding the usage of inert gases for their widespread practical application. Therefore, this work evaluated various regimes of hypoxic and anoxic nitrogen atmospheres for the control of two key stored-product pests, in laboratory and under field conditions in silos. *Sitophilus granarius* and *Callosobruchus chinensis* were selected as the tested species since they are important pests of grain/rice or legumes in Europe and Asia. Under laboratory conditions, we tested nitrogen (N_2) concentrations (from 95 to 100%) and exposure times (1–20 days) on the developmental stages of both pest species. In most developmental stages of *S. granarius* and *C. chinensis*, the shortest effective exposure was found for nitrogen concentration of 99%. Based on our laboratory tests, validation studies were subsequently carried out in semi-hermetic steel silos (25t) using continuous nitrogen saturation by on-site built swing pressure generators. It was found that a full control of all stages of *S. granarius* and *C. chinensis* was achieved in 11 days of nitrogen exposure, using concentrations ranging above 99% and below 100%. Our work shows that hypoxic nitrogen treatment can be effectively achieved in small steel silos under proper technological and environmental conditions.

Keywords: integrated pest management; controlled atmospheres; modified atmospheres; anoxia; hypoxia; stored product pests; phyto-sanitary treatment

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1. Introduction

Storage arthropods can cause multiple types of damage to agro-commodities during their storage [1], import and export [2]. Storage pests are also able to invade and spoil processed and packaged food [3–5]. In addition to economic quality damage, the infestation of commodities and processed foods by storage insects and mites is a problem in terms of their contamination of food with allergens [6,7]. Currently, storage pest risks are also increasing due to climatic temperature changes [8]. Another risk factor for effective pest control is the increasing resistance to phosphine, that is currently the most frequently used fumigant at a global scale [9–11]. The use of low temperatures [12], liquid nitrogen as freezing agents [13], alternative fumigants, ozone, or inert gases, are proposed as solutions [14,15]. In terms of societal requirements for residue-free exposure to products, yet effective pest control effects, inert gases and anoxic/hypoxic atmospheres are among the most promising [16,17]. Controlled atmospheres aim at creating a low-oxygen (hypoxia) or zero-oxygen (anoxia) environment that is lethal to pest insects and mites. Anoxic and hypoxic atmospheres, as eco-friendly pest control solution, can be applied using different

procedures or technologies in various types of stores and chambers [15,17,18], or commodity and food packaging [19–21]. Some types of anoxic and hypoxic atmospheres not only control pests, but they also may maintain the quality of the products [22,23]. The physiological effects of inert gases are in many aspects different from most other gaseous insecticides [24–26]. Thus, in addition to the ecological aspects, an advantage of inert gases is their effective action on pest populations that are already resistant to traditional insecticides. For example, Sakka et al. [27] and Agrafioti et al. [9], have recently demonstrated the high potential of hypoxic atmospheres to control populations of a number of phosphine-resistant storage pest species. In addition, controlled atmospheres are starting to be component of phyto-sanitary and phyto-quarantine treatment strategies [28,29].

Despite the above advantages of inert gases and controlled anoxic atmospheres, their use in practice may not be entirely straightforward, both from a technical and biological point of view. Regarding inert gases, and nitrogen in particular, a limited number of laboratory studies and practical applications are available. They describe their use in gas-tight chambers, laboratory experimental facilities and field stores and silos [30–37]. These works show the significant influence of different types of storage and the exposure regimes of inert gases, with respect to their effectiveness on different storage arthropod pest-species [38]. Commenting on the biological efficacy of anoxic atmospheres, Bailey and Banks [39] noted that the contemporary literature is apparently conflicting on the relative susceptibilities of various species, and the relative speed of action of N_2-oxygen or CO_2-oxygen mixtures. Even the more recent study by Liu [26] confirmed that there were considerable differences between stored product insect species and stages in susceptibility to low oxygen treatment. Not only pest species, but also various life stages of storage pests, may respond differently in terms of their susceptibility to various concentration of CO_2 and N_2 [26]. Contrary to what conventional intuition would suggest, Navarro [40] found that for certain storage pest species, a shorter controlled atmosphere (CA) exposure time is required if CA is composed of somewhat less than 100% nitrogen concentration. Therefore, depending on the particular pest species, effective nitrogen dosage and exposure must be maintained within a narrow range of effective concentrations [17]. The biological efficacy of CA is linked to the physical characteristics of the storage conditions and the technical characteristics of the stores. Differential gas tightness of various types of storage structures is among the key factors influencing procedures for the effective use of controlled atmospheres. Different construction types of commodity silos may also have different gas sorption characteristics, that influence the requirements for CA operational procedures. Technical structures and the method of filling inert gases affect the uniformity of their distribution within the treated objects [17,37]. Operational procedures of CA and exposure regimes can also be affected by the geographical location of the stores, as different areas have different thermal conditions. Areas with low ambient temperatures may require extended exposure times and increased gas consumption. Given the complexity of the practical usage of controlled atmospheres, Athanassiou [41] pointed out that there is still relatively little scientific documentation regarding usage inert gases for silo applications under field conditions. This implies that there is still a need to investigate the use of gases in different pest species and developmental stages, under different technical storage conditions and exposure regimes.

The present work was aimed at investigating the lethality of controlled hypoxic atmospheres on two species of primary pests of stored products, under laboratory conditions and in small silos. *Sitophilus granarius* L. (Curculionidae) and *Callosobruchus chinensis* L. (Chrysomelidae: Bruchinae) were selected as the tested species since they are important pests of grain/rice or legumes in Europe and Asia. Moreover, beetles of the genus *Sitophilus* and *Callosobruchus* are generally suitable model species for CA testing and validation, as they are among the less susceptible storage arthropods to anoxic atmospheres [42–45]. Based on the findings of Navarro [40], the specific objective of the laboratory part of the work was to determine whether the tested pest species belong to the group of storage pests for whose control it is optimal to reach concentrations around 99%, or to the second group

of species, for which the optimal control is 100% anoxia. Under laboratory conditions, we thus tested N_2 concentrations of gaseous nitrogen, on various developmental stages of *S. granarius* and *C. chinensis*. Jay [38] commented on the fact that there might not always be complete relevance between laboratory studies and the field application of CA to control stored-product insects. Therefore, based on our laboratory tests, validation studies were subsequently carried out in smaller semi-hermetic silos (25t) with continuous nitrogen saturation using on-site swing pressure nitrogen generators. Experiments were conducted in terms of the pest protection of imported agricultural commodities using standard shipping containers; therefore, tests were conducted in a set of small steel silos, each corresponding to the contents of one standard shipping container. The aim was to determine whether it was possible to achieve complete control of the tested pest species in 11 days under realistic operating conditions, or whether it was necessary to expose the pests for 21 days.

2. Materials and Methods

2.1. Tested Species and Cultures

The insects used for the study included two internally feeding stored-product Coleoptera species: *Sitophilus granarius* and *Callosobruchus chinensis*. Both populations (strains) tested were sensitive strains to commonly used insecticides, that were kept under laboratory conditions for at least 5 years. They were maintained in a rearing room at 25 °C and 75% RH. As feeding substrate was used wheat grain for *S. granarius* and mung beans for *C. chinensis*.

2.2. Laboratory Trials

2.2.1. Testing Apparatus

All pest species and their developmental stages were tested in a thermostatically controlled N_2 apparatus and the scheme is depicted in Figure 1. It is the modified version of the testing device proposed by Navarro et al. [45]. It consisted of 6 small plastic hermetic boxes (Lock and Lock HPL834, 2600 mL) in temperature 25 °C. These boxes were serially connected by transparent plastic hoses (PVC; diameter of 6/9 mm, Deutsch and Neumann GmbH; Hennigsdorf, Germany). The target nitrogen concentration (95%, 99% or 100%) was delivered from a pressurized metal cylinder (Linde Gas a.s.; Prague; Czech Republic) using a C200/2B-3SS outlet valve (Linde Gas a.s.; Czech Republic). A gas washing bottle containing an aqueous NaCl saturated solution was positioned between the metal cylinder and the initial small plastic box to humidity the nitrogen to 75% RH. The concentration of oxygen was determined using a GMH 3691 oxygen sensor (Greisinger Electronic GmbH; Regenstauf, Germany) connected behind the last experimental plastic box. The flow rate of nitrogen was measured using a Flow Sensor SFAB (Festo AG and Co. KG; Esslingen Germany).

2.2.2. Bioassay Experimental Protocol

In the experiments, the efficacy was validated on two species, *S. granarius* and *C. chinensis*. Validation of efficacy was performed for all developmental stages (adult, pupa, larva and egg). Each development stage was tested separately. The immature development stages were prepared according to the modified methodology of Aulicky et al. [37] (certified methodology for controlling the effectiveness of controlled atmospheres and fumigation in silos using bio-tests), as follows: (i) Eggs—The adults of both species were placed in a breeding container with substrate for 72 h. Then, the adults were removed and the substrate with the deposited eggs was used for further testing. (ii) Larvae and pupae—Adults were placed in the breeding container with substrate for 1 week. Then, the adults were removed and the substrate was stored at 25 °C in a thermo-chamber. For *S. granarius*, the substrate was used for larvae between 21–35 days, and for pupae between 35–48 days. For *C. chinensis*, the substrate was used for larvae between 14–21 days and for pupae between 28–35 days.

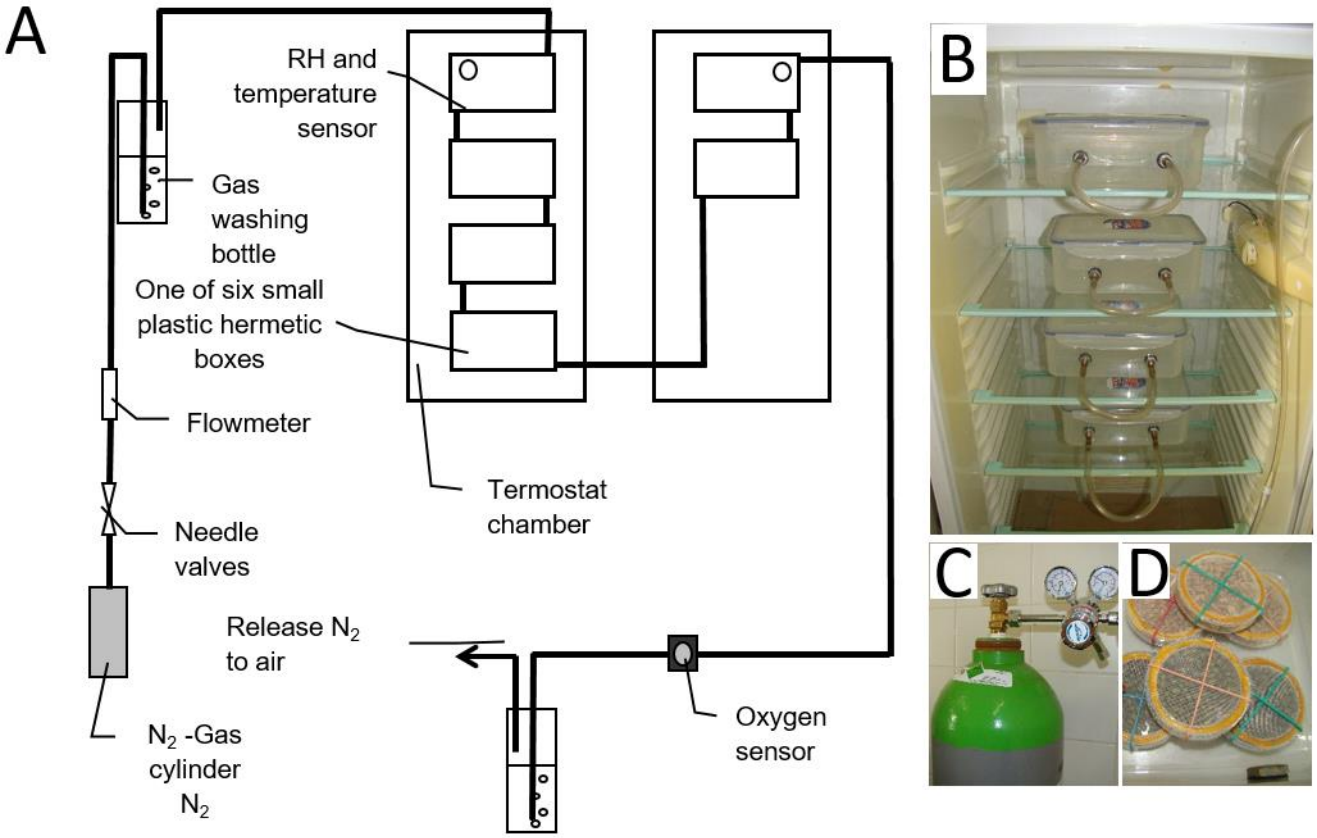

Figure 1. Testing apparatus for validation of nitrogen control atmospheres in laboratory conditions (**A**)—technical scheme; (**B**)—plastic boxes in a climatised thermostatic-chamber; (**C**)—source of N_2 (gas cylinders); (**D**)—bio-samples of pests and commodites in Petri dishes covered by mesh lid.

The tested stages were placed together with a substrate weighing 15 g (*S. granarius*—wheat grain and *C. chinensis*—mung beans) in Petri dishes (diameter 60 mm) that had a mesh lid (diameter of the hole in the lid 50 mm). Fifty adult individuals were placed into each dish. The age of adults of *S. granarius* was 7–14 days and *C. chinensis* 1–5 days.

All pest species and stages were enclosed and exposed in small plastic hermetic boxes; each box contained 8 petri dishes; one dish from each species and stage. In each treatment, 6 small plastic boxes were connected in series and the application of nitrogen from a pressurized metal cylinder was started. After reaching the target oxygen concentration, the pests were separately exposed for 1, 4, 8, 12, 16 or 20 days. After each exposure trial, the last small plastic box with the samples was always disconnected and the controlled atmosphere was vented. Subsequently, the treated box was placed in a thermostat with a temperature of 25 °C. Adult mortality was checked 4 days after the end of exposure. It was because it was previously described so called delayed mortality effect in some types of controlled atmospheres [44]. Check of efficacy for other developmental stages was performed using adult hatching. The inspections were carried out regularly once a week until the adults stopped hatching. The control samples were prepared in the same way as the experimental ones, but they were not exposed in boxes by hypoxic nitrogen atmosphere.

2.3. Field Silo Validation Trials

2.3.1. Silos and Source of N_2

Trials using N_2 were conducted in four identical steel silos with a capacity of 25 tons, fully loaded with a polished rice grain. Three silos were concurrently used as test silos filled with controlled nitrogen atmosphere (CA-N), whereas one silo was used as an untreated control without CA-N. The silos were shaded and thermally insulated by the roof and wall construction. The silos were not completely hermetic. However, they were sealed by

glue (metal silo seals) and rubber (silo top and bottom covers) and they were equipped with a gastight press plenum through which the nitrogen was introduced. Due to these additional adaptations, we called the silos "semi-hermetic" further in the text. The silos were located inside the building of a food processing facility located in the Czech Republic (Podravka-Lagris a.s., south Moravia). The treated semi-hermetic silo bins included the discoid panel with a hole and plug for nitrogen filling at the top of the silo (Figure 2D). The hermetic discoid panel—located at the bottom of the silo—has a hole with a plug for nitrogen/oxygen concentration measurement (Figure 2B). For further technical description of semi-hermetic silos, see Aulicky et al. [37]. The nitrogen (N_2) controlled atmosphere was produced from two on-site installed sets based on Swing Pressure Nitrogen Generators (N_2—NITRO Source PSA, Parker) (Figure 2A). Each set consisted of the following units: compressor, air receiver, adsorption dryer, nitrogen buffer vessel, low-pressure N_2 storage unit and piping.

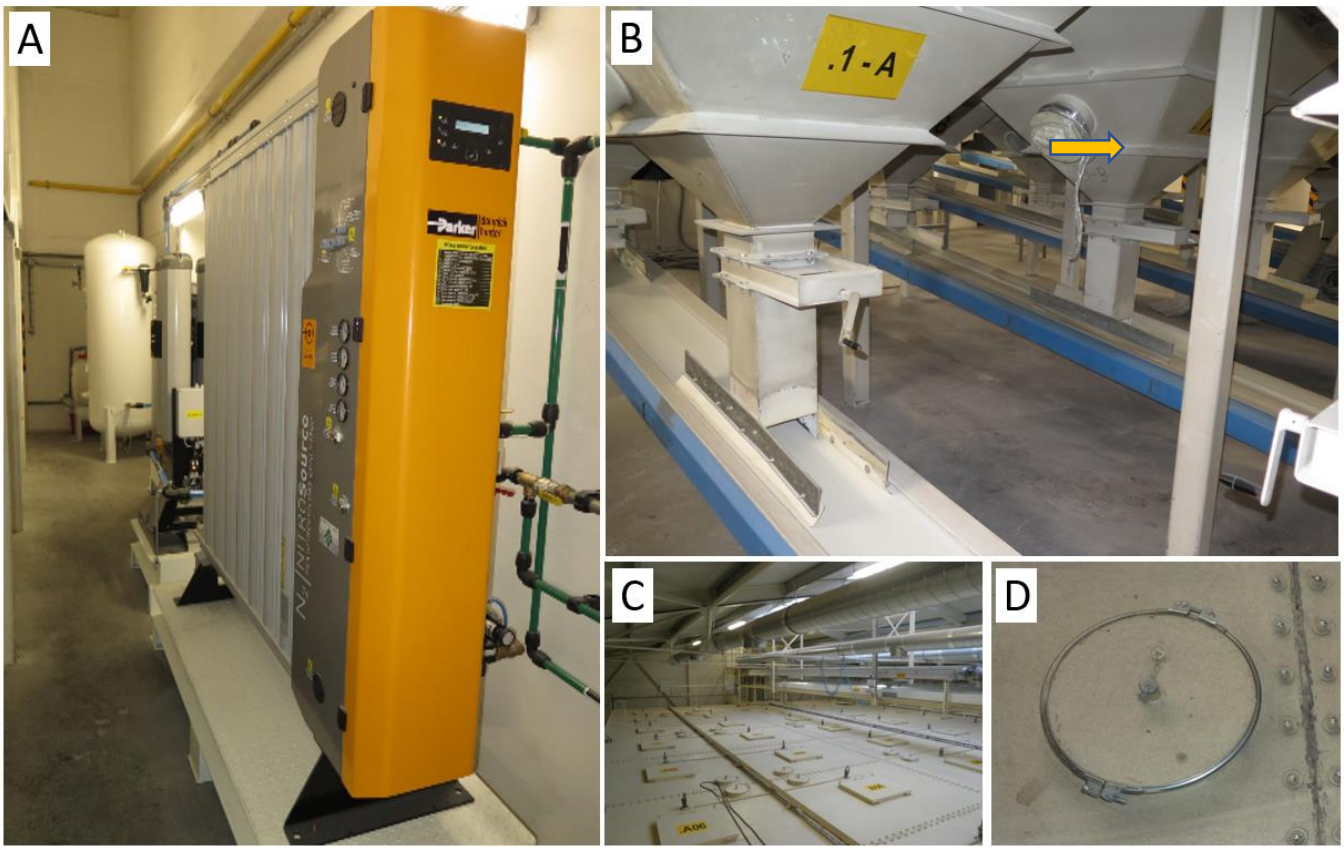

Figure 2. Visualisation of the validation of biological efficacy of nitrogen-controlled atmospheres in silos. (**A**)—the on-site- build pressure swing nitrogen generator (N_2—NITRO Source PSA—Parker) consisting of compressor, air receiver, adsorption dryer, nitrogen buffer vessel, low pressure N_2 storage unit and piping; (**B**)—hermetic removable discoid panel at the bottom of the silo with a hole and plug for nitrogen/oxygen measures; (**C**)—top board of silo bins with rectangular opening for loading the commodity and discoid panels; (**D**)—detail of a hermetic discoid panel with plug for nitrogen filling at the top of the silo.

2.3.2. Temperature, Humidity and Oxygen Level

Oxygen concentration measurements were performed with a Dräger X-am 7000 portable detector (Dräger, GmbH, Stuttgart, Germany). Temperature and RH were measured during treatment in a selected cell at the top using a TinyTag Ultra 2 digital data logger (TinyTag Ultra 2; Gemini Data Loggers Ltd., Chichester, UK). During the shorter exposure period (10 + 1 day), the mean temperature was 19.2 ± 0.1 °C (range: 18.8–20.8 °C) and RH

was 52.1 ± 0.1 % (48.0–52.8 %), and during the longer exposure period (20 + 1 day), the mean temperature was 21.0 ± 0.1 °C (20.3–21.8 °C) and RH was 50.0 ± 0.1 % (34.3–50.8%).

2.3.3. Bioassay Exposure

Validation of the effectiveness of the controlled atmosphere with nitrogen was carried out in four metal silos (each capacity of 25 tonnes) containing polished rice-silos are described above. All developmental stages (adults, pupae, larvae and eggs) of the two primary pest species (*S. granarius* and *C. chinensis)* were used in the tests. Biological samples were prepared following the same procedure as for the laboratory tests (Section 2.2.2). In the case of adults, 20 individuals were used per dish. Samples were located in two positions in each silo. One group of samples was placed at the top and the other at the bottom of the silo unit. A total of 32 dishes (4 dishes for each species and developmental stage) were placed at each position in the case of CA-N treated cells. A total of 48 dishes (6 dishes for each species and developmental stage) were placed in the control silo unit without CA-N treatment. Treatments were based on N_2 purging and N_2 maintenance phases. After the start of nitrogen application, there was a so-called purging or filling phase, which lasts 24 h. The target oxygen concentration was below 1% through the entire silo profile, including the bottom of the silo unit. Reaching this concentration, an exposure period of 10 or 20 days was calculated. Thus, the total exposure periods were either 11 days (1 day N_2 purging; 10 days of N_2 maintenance) or 21 days (1 day N_2 purging; 20 days of N_2 maintenance). During the treatment period, the oxygen concentration at the bottom of all silos was checked at regular intervals. At the end of each of both exposure periods, the differentially exposed samples were removed and transferred to the laboratory. The procedure for evaluating the biological efficacy of CA-N on the tested pest species and developmental stages was the same as described above for the laboratory trials.

2.4. Statistical Procedures

Data from the laboratory test were analyzed as a multifactorial experiment design for each species separately. In adults, concentration and exposure times were assessed for survival and mortality. For the other developmental stages (eggs, larvae and pupae), concentration, exposure time and developmental stage for adult pupation were evaluated. For statistical purposes, each species was evaluated separately. Exposure time and concentration were entered as factors in the ANOVA. Concentrations also included controls without nitrogen. Each exposure time included separate evaluation of two concentrations. Data were transformed and evaluated using parametric multi-factorial ANOVA test (Statistica statistical software, version 12; StatSoft CR s.r.o., Prague, Czech Republic). The homogeneity of the groups was further evaluated using the Tukey—Kramer HSD post-hoc test at the 0.05 level of significance.

3. Results

3.1. Laboratory Trial

Laboratory results showed that the effectiveness of three different concentrations of nitrogen atmospheres varied for the tested pest species and their developmental stages, depending on the duration of exposure. Parameters of ANOVA for the main effects of controlled atmospheres in *Sitophilus granarius* and *Callosobruchus chinensis* are summarized in Table 1. Table 2 shows the effect of various exposure times on adult mortality of *S. granarius* adults at three concentrations of nitrogen atmosphere. In the controlled atmosphere of 100% N_2, the complete adult mortality was achieved at an exposure time of 8 days, whereas for 99% N_2 atmosphere it required 12 days' CA-N exposure for the same biological efficacy. The lowest used concentration of nitrogen atmosphere (i.e., 95% N_2) failed to deliver complete adult mortality in even 20 days. Table 3 presents data on the emergence of adults from previously treated grain containing eggs, larvae, or pupae of *S. granarius* by two concentration of nitrogen atmosphere. Controlled atmosphere of 95% CA-N did not lead to suppression of all treated stages even within 20 days, except one

case regarding pupae treatment. Both N_2 concentrations (i.e., 99 and 100%) completely suppressed the development of eggs and larvae equally rapidly. The complete inhibition of egg and larval emergence was achieved at 12 day exposure time at both concentrations. However, higher concentration of N_2 (100%) requires longer exposure time (16 days) to suppress development of pupae than the slightly lower N_2 concentration (99%) (12 days).

Table 1. Parameters of ANOVA for main effects related to mortality of the exposed adults of *Sitophilus granarius* (df = 180) and *Callosbruchus chinensis* (df = 180) or to *S. granarius* (df = 315) and *C. chinensis*—(df = 315). adult emergence following their previous N_2 exposure in sub-adult stages (egg, larva and pupa).

Species	Stages	Source	*df*	*F*	*p*
S. granarius	Adults	Exposure time	5	284.9	<0.001
		Concentration	5	3042.4	<0.001
		Exposure time × Concentration	25	77.8	<0.001
C. chinensis	Adults	Exposure time	5	981.2	<0.001
		Concentration	5	201.9	<0.001
		Exposure time × Concentration	25	77.1	<0.001
S. granarius	Eggs, larvae, pupae	Exposure time	6	696.2	<0.001
		Concentration	2	127.9	<0.001
		Stage	2	4.2	<0.016
		Exposure time × Concentration	12	15.5	<0.001
		Exposure time × Stage	12	11.4	<0.001
		Concentration × Stage	4	19.6	<0.001
		Exposure time × Concentration × Stage	24	2.1	<0.003
C. chinensis	Eggs, larvae, pupae	Exposure time	6	711.0	<0.001
		Concentration	2	150.8	<0.001
		Stage	2	104.2	<0.001
		Exposure time × Concentration	12	7.8	<0.001
		Exposure time × Stage	12	24.4	<0.001
		Concentration × Stage	4	54.6	<0.001
		Exposure time × Concentration × Stage	24	16.1	<0.001

Table 2. Laboratory efficacy (% of mean adult mortality; Av. % ± SE) of six exposure times (ranging from 1 to 20 days) and three concentrations (ranging from 95% to 100% N_2) nitrogen-based controlled atmospheres on the exposed adults of grain weevil (*Sitophilus granarius*). (Different letters indicate statistically significant differences between variables).

Exposure Time		Concentration		
		100% N_2	99% N_2	95% N_2
1 day	Exposed	73.7 ± 2.0 Aa	15.0 ± 2.1 Ba	4.3 ± 1.4 Ca
	Control	1.0 ± 1.0 Ab	0.7 ± 0.4 Ab	1.3 ± 1.0 Aa
4 days	Exposed	93.7 ± 2.0 Ac	79.3 ± 1.8 Bc	10.3 ± 2.3 Ca
	Control	2.3 ± 1.1 Ab	0.7 ± 0.4 Ab	3.0 ± 1.1 Aa
8 days	Exposed	100.0 ± 0.0 Ac	95.3 ± 1.4 Ad	20.7 ± 2.8 Bb
	Control	4.0 ± 1.6 Ab	4.7 ± 2.0 Ab	4.7 ± 1.4 Aa
12 days	Exposed	100.0 ± 0.0 Ac	100.0 ± 0.0 Ad	32.3 ± 4.3 Bc
	Control	4.0 ± 1.6 Ab	6.0 ± 1.8 Aab	6.0 ± 1.4 Aa
16 days	Exposed	100.0 ± 0.0 Ac	100.0 ± 0.0 Ad	65.7 ± 5.5 Bd
	Control	4.70 ± 1.5 Ab	6.7 ± 1.4 Aab	10.0 ± 1.9 Aa
20 days	Exposed	100.0 ± 0.0 Ac	100.0 ± 0.0 Ad	82.3 ± 2.0 Be
	Control	4.7 ± 1.5 Ab	6.7 ± 1.4 Aab	10.3 ± 2.2 Aa

Table 3. Laboratory efficacy (Average No. of adults emerged following their treatment in subadult stages; Av. No. ± SE) of six exposure times (ranging from 1 to 20 days) and three concentrations (ranging from 95% to 100% N_2) nitrogen-based controlled atmospheres on eggs, larvae and pupae of grain weevil (*Sitophilus granarius*). (Different letters indicate statistically significant differences between variables).

Development Stage	Exposure Time	Concentration		
		100% N_2	99% N_2	95% N_2
Eggs	1 day	27.8 ± 1.8 Aa	32.7 ± 2.8 Aa	41.8 ± 1.6 Ba
	4 days	22.7 ± 1.9 Aa	29.0 ± 2.6 ABab	35.5 ± 2.4 Bab
	8 days	19.5 ± 1.1 Aa	17.2 ± 3.4 Ab	33.3 ± 1.1 Bab
	12 days	0.0 ± 0.0 Ab	0.0 ± 0.0 Ac	26.8 ± 1.3 Bb
	16 days	0.0 ± 0.0 Ab	0.0 ± 0.0 Ac	17.8 ± 2.7 Bbc
	20 days	0.0 ± 0.0 Ab	0.0 ± 0.0 Ac	10.5 ± 1.3 Acd
	Control	41.0 ± 1.5 Ac	38.7 ± 2.7 Aa	45.5 ± 1.7 Aa
Larvae	1 day	37.5 ± 2.6 Aa	47.2 ± 1.4 Aa	39.2 ± 2.7 Aa
	4 days	22.0 ± 2.1 Ab	23.7 ± 2.4 Ab	35.8 ± 2.7 Ba
	8 days	2.5 ± 0.6 Ac	4.3 ± 1.2 Ac	25.8 ± 2.6 Bab
	12 days	0.0 ± 0.0 Ac	0.0 ± 0.0 Ac	21.5 ± 1.8 Bbc
	16 days	0.0 ± 0.0 Ac	0.0 ± 0.0 Ac	10.2 ± 1.6 Acd
	20 days	0.0 ± 0.0 Ac	0.0 ± 0.0 Ac	3.8 ± 1.5 Ad
	Control	46.5 ± 1.8 Aa	54.5 ± 1.3 Aa	45.7 ± 3.6 Aa
Pupae	1 day	45.2 ± 5.6 Aad	44.3 ± 1.9 Aa	42.0 ± 2.1 Aa
	4 days	37.5 ± 1.9 Aa	29.8 ± 3.2 Ab	35.8 ± 2.1 Aa
	8 days	18.5 ± 3.8 Ab	11.2 ± 2.4 Ac	20.8 ± 1.7 Ab
	12 days	3.8 ± 1.9 Abc	0.0 ± 0.0 Ac	18.3 ± 2.9 Bbc
	16 days	0.0 ± 0.0 Ac	0.0 ± 0.0 Ac	6.7 ± 0.9 Acd
	20 days	0.0 ± 0.0 Ac	0.0 ± 0.0 Ac	0.0 ± 0.0 Ad
	Control	52.2 ± 4.7 Ad	47.2 ± 1.9 Aa	45.3 ± 2.6 Aa

Table 4 shows adult mortality and Table 5 shows the emergence of adults from previously treated eggs/larvae/pupae of *C. chinensis* following their exposure to three concentration of controlled nitrogen atmosphere (CA-N). For 99% and 100% CA-N the complete *C. chinensis* adult mortality was reached in 8 days, whereas 95% CA-N required 12 days of exposure. However, the latter was associated with 100% mortality in the untreated control since *C. chinensis* adults are short-lived. Controlled nitrogen atmosphere (CA-N) achieved more rapid action (12 days) at a slightly lower CA-N concentration (99% N_2) than at the highest concentration (100% N_2) (16 days) in all immature stages except pupae. At a CA-N concentration of 99% N_2, 100% larval mortality was attained at the 12 day exposure, while at a higher concentration up to the 16 day exposure. Similar to *S. granarius*, the lowest used concertation of CA-N (95%) did not lead to the suppression of all treated stages of *C. chinensis* within 20 days of exposure, except in one case regarding pupae treatment.

3.2. Field Trials in Silos

The initial N_2 purging phase of nitrogen into the commodity-filled silos required 24 h for both types of exposures, i.e., 1 + 10 or 1+ 20 days. The average nitrogen consumption was 148.5 L*min^{-1} in the shorter exposure time and 147.3 L*min^{-1} in the longer exposure time. From Figures 3 and 4, it is apparent that in all silos oxygen concentrations below 2% was achieved (at the bottom of each silo unit) within 12 h, and it took a further 12 h to achieve oxygen concentrations below 1%. In the subsequent saturation and maintenance phase, despite slight diurnal fluctuations in temperature and atmospheric pressure, oxygen concentrations between 0% and 1% were maintained for both the 10 day exposure (Figure 3) and the 20 day exposures (Figure 4).

Table 4. Laboratory efficacy (% of mean adult mortality; Av. % ± SE) of six exposure times (ranging from 1 to 20 days) and three concentrations (ranging from 95% to 100% N2) nitrogen-based controlled atmospheres on the exposed adults of adzuki bean weevil (*Callosobruchus chinensis*). (Different letters indicate statistically significant differences between variables).

Exposure Time		Concentration		
		100% N_2	99% N_2	95% N_2
1 day	Exposed	97.0 ± 1.7 Aa	40.0 ± 2.1 Ba	27.7 ± 3.8 Ca
	Control	76.3 ± 3.7 Ab	26.0 ± 1.8 Bb	18.3 ± 3.3 Bb
4 days	Exposed	100.0 ± 0.0 Aa	84.0 ± 2.3 Bc	44.0 ± 3.3 Cc
	Control	87.7 ± 3.2 Ac	78.7 ± 1.4 Ac	36.0 ± 3.6 Bbc
8 days	Exposed	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	90.0 ± 2.8 Bd
	Control	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	87.0 ± 2.4 Bd
12 days	Exposed	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	100.0 ± 0.0 Ae
	Control	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	100.0 ± 0.0 Ae
16 days	Exposed	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	100.0 ± 0.0 Ae
	Control	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	100.0 ± 0.0 Ae
20 days	Exposed	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	100.0 ± 0.0 Ae
	Control	100.0 ± 0.0 Aa	100.0 ± 0.0 Ad	100.0 ± 0.0 Ae

Table 5. Laboratory efficacy (Average No. of adults emerged following their treatment in subadult stages; Av. No. ± SE) as of six exposure times (ranging from 1 to 20 days) and three concentrations (ranging from 95% to 100% N_2) nitrogen-based controlled atmospheres on eggs, larvae of adzuki bean weevil (*Callosobruchus chinensis*). (Different letters indicate statistically significant differences between variables).

Development Stage	Exposure Time	Concentration		
		100% N_2	99% N_2	95% N_2
Eggs	1 day	9.2 ± 1.2 Aa	18.7 ± 1.2 a	34.7 ± 1.6 Bad
	4 days	5.2 ± 1.4 Aa	16.5 ± 2.2 Ba	29.5 ± 1.8 Cab
	8 days	6.0 ± 1.3 Aa	9.5 ± 1.8 Aab	22.7 ± 1.8 Bb
	12 days	3.7 ± 1.4 Aa	0.0 ± 0.0 Ab	10.0 ± 1.3 Ac
	16 days	0.0 ± 0.0 Aa	0.0 ± 0.0 Ab	7.7 ± 1.1 Ac
	20 days	0.0 ± 0.0 Aa	0.0 ± 0.0 Ab	1.0 ± 0.5 Ac
	Control	21.3 ± 2.7 Ab	33.00 ± 0.9 Bc	43.3 ± 2.1 Bd
Larvae	1 day	54.3 ± 4.5 Aa	29.0 ± 1.3 Ba	37.0 ± 2.3 Ba
	4 days	22.5 ± 2.3 Ab	21.7 ± 1.3 Aa	30.0 ± 1.8 Aab
	8 days	18.7 ± 2.3 ABb	8.7 ± 3.9 Ab	21.5 ± 0.4 Bbc
	12 days	1.5 ± 0.62 Ac	0.0 ± 0.0 Ab	14.0 ± 1.6 Bc
	16 days	0.0 ± 0.0 Ac	0.0 ± 0.0 Ab	6.7 ± 1.0 Acd
	20 days	0.0 ± 0.0 Ac	0.0 ± 0.0 Ab	5.8 ± 1.1 Acd
	Control	63.2 ± 3.5 Aa	55.7 ± 2.1 ABc	51.5 ± 1.7 Be
Pupae	1 day	31.5 ± 5.3 Aa	15.2 ± 1.4 Ba	29.3 ± 0.9 Aa
	4 days	47.2 ± 3.6 Ab	6.3 ± 0.7 Bab	25.0 ± 1.7 Ca
	8 days	11.8 ± 1.9 Ac	2.5 ± 0.8 Ab	22.8 ± 2 Ba
	12 days	0.8 ± 0.5 Ad	0.0 ± 0.0 Ab	8.5 ± 0.9 Ab
	16 days	0.5 ± 0.4 Ad	0.0 ± 0.0 Ab	3.2 ± 0.6 Ab
	20 days	0.0 ± 0.0 Ad	0.0 ± 0.0 Ab	0.00 ± 0.0 Ab
	Control	52.7 ± 2.8 Ab	30.8 ± 4.7 Bc	49.3 ± 1.3 Ac

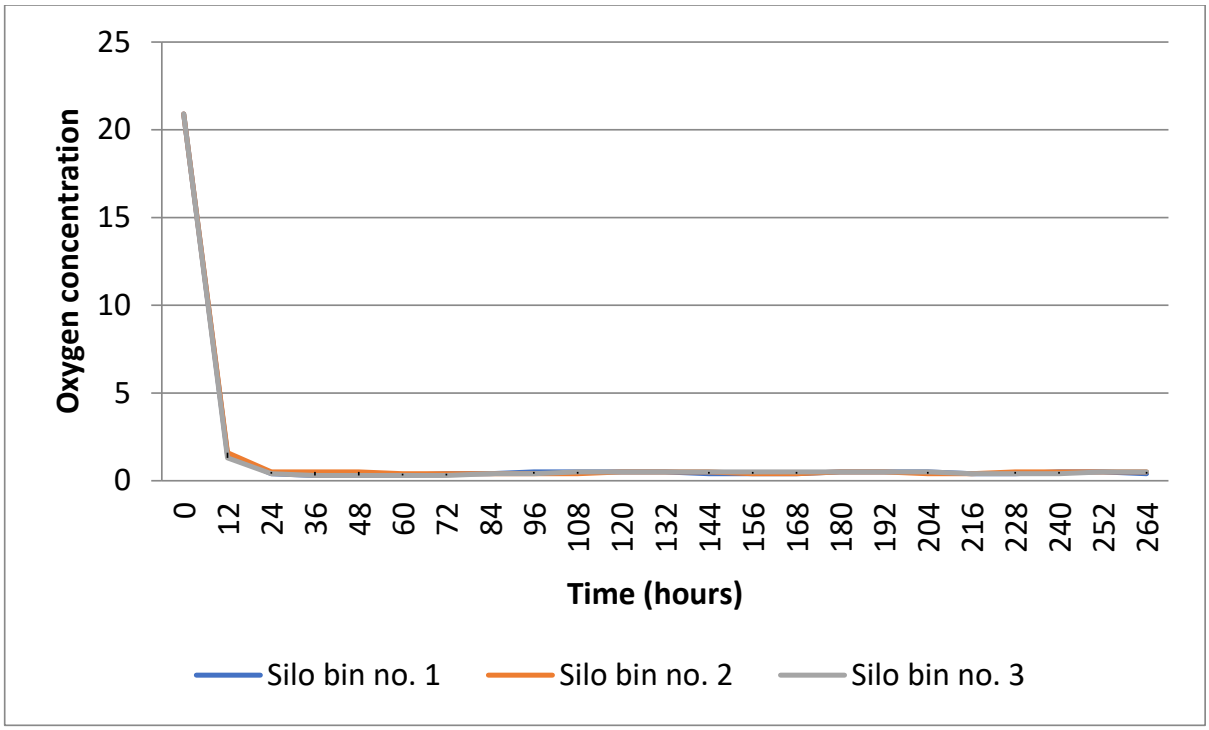

Figure 3. Oxygen concentration during field test—10 + 1 days of exposure (n = 3 silo units); 1 day N_2 purging; 10 days of full N_2 concentration exposure. The average oxygen concentration after initial 12 h nitrogen purging was 1.5 ± 0.1%; the average oxygen concentration for the 10 days of nitrogen maintenance was 0.4 ± 0.0%.

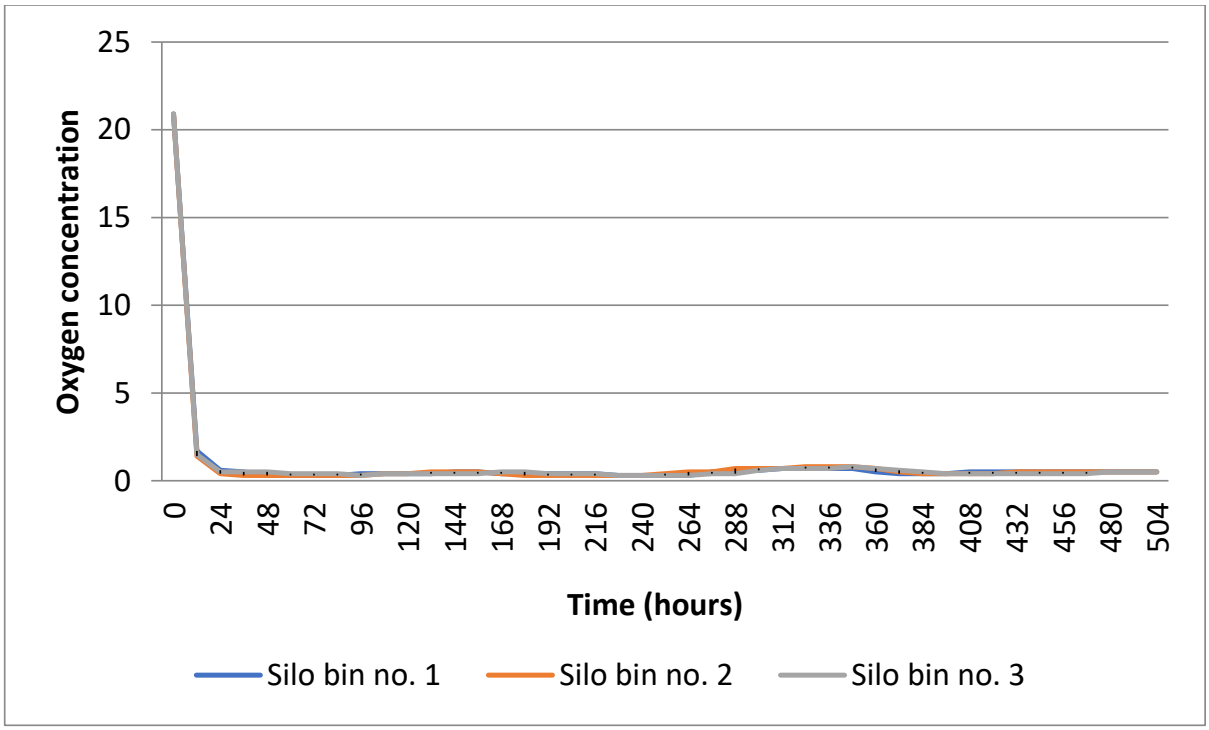

Figure 4. Oxygen concentration during field test—20 + 1 days of exposure (n = 3 silo units); 1 day N_2 purging; 20 days full N_2 concentration exposure. The average oxygen concentration after initial 12 h nitrogen purging was 1.5 ± 0.1%; the average oxygen concentration for the 20 days of nitrogen maintenance was 0.5 ± 0.0%.

The data summary on the mortality of the controlled nitrogen's atmospheres (below 1% of oxygen) on adults of *S. granarius* and *C. chinensis* in metal silos, under two exposure regimes of 10 or 20 days, can be found in Table 6. Table 7 shows the efficacy of the tested nitrogen atmospheres on eggs, larvae and pupae of both tested species. All field validation tests, carried out in small semi-hermetic silos, showed 100% mortality (Table 6) or 100% development suppression (Table 7) in both exposure regimes and all tested species and their developmental stages under given conditions.

Table 6. Field efficacy (% of adult mortality; Av. % ± SE) of controlled nitrogens atmospheres (below 1% of oxygen) on adults of the tested species *(S. granarius, C. chinensis*) in metal silos under two exposure regimes 11 (1 day N_2 purging; 10 days full N_2 concentration exposure) or 21 days. For each treated silo units (n = 3 silos) 4 bio-assay-dishes with insects were used.

Species	Sample	n	Exposure Time	
			11 Days	21 Days
S. granarius	TS Upper	3	100.0 ± 0.0	100.0 ± 0.0
	TS Lower	3	100.0 ± 0.0	100.0 ± 0.0
	Control—Lower position	1	6.7 ± 2.8	1.7 ± 1.1
C. chinensis	TS Upper	3	100.0 ± 0.0	100.0 ± 0.0
	TS Lower	3	100.0 ± 0.0	100.0 ± 0.0
	Control—Lower position	1	96.7 ± 1.1	99.2 ± 0.8

Table 7. Field efficacy (Average No. of adults emerged following their treatment in subadult stages; Av. No. ± SE) of controlled nitrogens atmospheres (below 1% of oxygen) on eggs, larvae and pupae of the tested species in metal silos under two exposure regimes of 11 or 21 days. For each treated silo units (n = 3 silos) 4 bio-assay-dishes with insects were used.

Species	Stage	Sample	n	Exposure Time	
				11 Days	21 Days
S. granarius	Pupae	TS Upper	3	0.0 ± 0.0	0.0 ± 0.0
		TS Lower	3	0.0 ± 0.0	0.0 ± 0.0
		Control—Lower position	1	4.7 ± 0.5	9.3 ± 1.5
C. chinensis		TS Upper	3	0.0 ± 0.0	0.0 ± 0.0
		TS Lower	3	0.0 ± 0.0	0.0 ± 0.0
		Control—Lower position	1	29.7 ± 1.9	22.0 ± 3.8
S. granarius	Larvae	TS Upper	3	0.0 ± 0.0	0.0 ± 0.0
		TS Lower	3	0.0 ± 0.0	0.0 ± 0.0
		Control—Lower positon	1	5.2 ± 0.6	23.0 ± 1.9
C. chinensis		TS Upper	3	0.0 ± 0.0	0.0 ± 0.0
		TS Lower	3	0.0 ± 0.0	0.0 ± 0.0
		Control—Lower position	1	16.3 ± 1.4	36.3 ± 5.6
S. granarius	Eggs	TS Upper	3	0.0 ± 0.0	0.0 ± 0.0
		TS Lower	3	0.0 ± 0.0	0.0 ± 0.0
		Control—Lower position	1	6.8 ± 1.0	9.3 ± 1.3
C. chinensis		TS Upper	3	0.0 ± 0.0	0.0 ± 0.0
		TS Lower	3	0.0 ± 0.0	0.0 ± 0.0
		Control—Lower position	1	124.3 ± 27.9	33.0 ± 9.7

4. Discussion

Good technological practice for commodity storage on farms, and their onward export and import, requires the availability of robust, rapid and environmentally safe pest control procedures. Compared to toxic fumigants, the use of modified atmospheres (= controlled atmospheres or hermetic storage) is considered challenging in terms of the requirements

for relatively long exposure times [16,17,46,47]. For a long-term storage of agricultural commodities at large farms or national strategic reserves facilities, the length of exposure to controlled atmospheres used in silos is usually not a major technological constraint [26]. However, the length of exposure to controlled atmospheres can play a significant role in the short-term storage of commodities on small farms with limited storage capacity, in ports [39], or in commercial organizations that process agro-commodities (cereals, rice, legumes, dry/dried fruits) into small food packages for retail sales [44,48]. For such companies, minimizing concentrations and exposure duration while achieving high efficacy is an essential constraint for the implementation of controlled atmospheres for routine practical use. This work therefore addressed the problem of optimization of the length of exposure by hypoxic nitrogen atmospheres for the control of two important storage pests, under laboratory and small-silos conditions.

4.1. Laboratory Trials

In the laboratory part of our work, new biological data were obtained regarding the biological efficacy of controlled nitrogen atmospheres on *C. chinensis* and *S. granarius* species under different exposure lengths (1–20 days) and different concentrations (95–100%). From a practical point of view, an important finding of our work was that for most of the tested exposures up to 20 days, the concentrations 99% and 100% nitrogen were highly effective for the control of all developmental stages (eggs, larvae, adult pupae) of both species. In contrast, at 95% nitrogen concentrations, 100% mortality was not achieved for all life stages of both species tested for most of the tested exposure times. The published work on laboratory exposures of *C. chinensis* and *S. granarius* with controlled atmospheres is relatively sparse [49–55]. As a result, a direct comparison of our results with data from other authors is also relatively limited. Cui et al. [49] investigated survival, development time delay and metabolomics changes in larvae (4^{th} instar) of *C. chinensis* under two hypoxia regimes (2% O_2 = hypoxia or 2% O_2 + 18% CO_2 = hypoxia/hypercapnia). They found that the development of *C. chinensis* was significantly suppressed by both hypoxia conditions and showed profound differences in metabolites between the treatment and control groups. Cui et al. [49] concluded that *C. chinensis* has high tolerance to hypoxia since some proportion of 4th-instar larvae survived (i.e., emerged as adults after 24 days) more than 20 days under hypoxia/hypercapnia. In our experiments, we achieved 100% mortality of *C. chinensis* larvae when exposed in nitrogen atmosphere 99% for 12 days. However, the larval survival and adult emergence of *C. chinensis* was observed for all tested exposure times at 95% nitrogen atmosphere. Regarding *S. granarius*, Lindgren and Vincent [50] reported 95% mortality of *S. granarius* adults after 5.3 day exposure by 100% nitrogen exposure (70 °F and 60–70% RH). Adler [54] observed 99% mortality from 10 strains of *S. granarius* in 6.6 days (20 °C and 75–99% nitrogen and 1% oxygen). The results by Adler [54,55] are comparable with the results obtained later by Conyers and Bell [56] on the sensitive and resistant strains of *S. granarius* (99.3–100% mortality, in 8 days at 99% nitrogen atmosphere-50% RH and 20 °C), and with the results obtained in our study (100 % mortality in 8 days at 100% nitrogen atmosphere, in 12 days at 99% nitrogen atmosphere).

Although direct comparison with available literature on the tested species is limited, it is still interesting to compare our results with abundant publications concerning the effectiveness of controlled atmospheres on the related pest species. They mainly include *Callosbruchus maculatus* [44,57–62], *Acanthoscelides obtectus* [57,63], *Sitophilus zeamais* [34], and *S. oryzae* [33,35]. Comparisons of data on *C. chinensis* and *S. granarius* with data on related species allow further generalization of the biological effects of inert gases on pests [24,64], and thus the construction of generally applicable atmosphere-controlled technologies and procedures for stored product pests [17]. Our laboratory data show that *C. chinensis* and *S. granarius* adults were the most susceptible developmental stages, which is in agreement with many findings obtained by other authors for several species of storage pests. For example, Hashem et al. [63] found that hypoxia/hypercapnia effects were more severe in adult bruchids *A. obtectus*, which led to 100% mortality after 3 days of exposure, but the

full hatchability suppression required 7 days. However, for *S. zeamais*, Williams et al. [35] found that not only adult stages but also eggs were more sensitive to anoxia than larval and pupal stages. The comparison of our data with the previously published results also suggested that the tested species of internal-feeding pests might be less sensitive to controlled atmospheres compared to some species of externally-feeding pests, such as *Tribolium* sp. and *Oryzaephilus* sp. [37,44,56,65,66]. For example, Sakka et al. [44] found that *C. maculatus* is more tolerant to nitrogen atmosphere than *T. castaneum*. They hypothesized that the difference in their sensitivity could be because the immature development of *C. maculatus* occurs in the inner part of the seeds, where nitrogen penetration may be more gradual, and the *T. castaneum* individuals are more directly exposed to nitrogen. However, several authors have shown that some externally developing pests, such as *Cryptolestes* spp. and *Trogoderma* genera [29,44,46,56,66], can exhibit tolerance to controlled hypoxic/anoxic atmospheres. For example, Conyers and Bell [56] found that adults of *C. ferrugineus* appeared to be most tolerant to an atmosphere with 1% oxygen, followed by *S. granarius*, while adults of *O. surinamensis* were the most sensitive. Moncini et al. [33] reported that an atmosphere containing 98.5% N_2 caused the complete mortality of *S. oryzae* adults after 3 days of exposure on wheat kernels, whereas the complete mortality of *T. confusum* adults required 7 days on flour. This is surprising, because it was previously reported [65] that externally developing *T. confusum* was very sensitive to nitrogen atmosphere. In this case, the question is whether sensitivity may be influenced not only by pest species, but also by the type of food commodity, such as grain or flour [33]. All the discrepancies found in the above-mentioned published works suggest that the affiliation of a particular species of a storage pest to an ecotype, with development inside or outside the seed commodity, is not a completely reliable predictor of its sensitivity or tolerance to anoxic/hypoxic atmosphere. In addition, the complex biotic and abiotic relationships discussed above indicate that practical procedures and exposure regimes for the use of a controlled atmosphere in practice should be more or less specific to each pest species.

Another practical objective of our laboratory work was to explore the mortality-concertation relationship in the tested species for the 95%-100% range of atmospheric nitrogen concentrations. Navarro and Navarro [17] pointed out that effective nitrogen dosage and exposure must be relatively precise and within a certain range of concentrations, because mortality may not have a positive linear relationship with increasing nitrogen concentration over the entire range of values. The basis for this caveat was previous laboratory experiments conducted by Navarro [40]. He found that, contrary to intuitive expectation, the necessary exposure time for some species increases when the atmospheric oxygen concentration approaches zero. In other species, however, 100% anoxia may be more effective than various levels of hypoxia. For example, Navarrro [40] described that *Ephestia cautella* pupae and *Tribolium castaneum* adults reacted to increasing concentrations of N_2 in a similar pattern, whereas *S. oryzae* adults reacted in a different way. The lower the oxygen concentration was, the shorter the exposure time needed to produce 95% mortality of *E. cautella* pupae and *T. castaneum*. However, *S. oryzae* adults showed greater sensitivity at 1% oxygen than at the zero level of oxygen concentration. In our study, we found that responses to different oxygen concentrations differed not only between the two species tested, but also among developmental stages within a species. In *S. granarius*, lethal exposure was shorter at 100% nitrogen concentration than at the lower concentration (99%), but the opposite was true for the pupal stage of this species. For the species *C. chinensis*, the exposure time leading to 100% mortality was the same for the 99% and 100% concentrations, whereas for the immature stage, the exposure time leading to 100% mortality was higher for the lower of the two concentrations tested, 99%.

4.2. Field Validations

The required effective pest control exposures of commodities to hypoxic atmospheres can exceed one month, depending on the technological and environmental conditions in commodity stores and silos [35]. However, such long exposure times may not be acceptable

for many companies for operational and economic reasons [44]. Users of controlled atmosphere usually balance between minimizing the length of the hypoxic exposure of infested commodities and maximizing its biological efficacy on pests. Unfortunately, practitioners frequently do not have detailed information to optimize these control processes for many pest species when controlling them in small silos. Most practical validations are either from large silos [67,68], laboratory small silos [34], mini-silos [35], glass desiccators [56] or hermetic chambers [44,48]. Published data regarding the use of controlled atmospheres for the treatment of commodities in smaller silos (25t) is lacking, or is very limited [35,37,69]. At the same time, detailed technological information is very important because the treatment of small silos is challenging; even small gas leaks cause a significant reduction in the concentration of inert gases [17,70]. For potential users, compensating for nitrogen leakage from silos by continuously saturating the silo-atmosphere with inert gases may seem economically and operationally demanding. However, our fieldwork has shown that good results can be achieved even in small metal silos in the normal operating conditions of a commercial commodity store (Podravka-Lagris, CZ). Our validations in these silos demonstrated that these types of silos could be saturated by the 98% nitrogen within 12 h, and a target hypoxic concentration above 99% can be well achieved within 24 h. It was further demonstrated that under a period of 10–20 days, a relatively stable level of over 99% nitrogen can be maintained; oxygen concentration oscillating between 0.3–0.7%. However, it should be noted that the treated silos were located indoors. Thus, the large fluctuations in temperature and the effects of direct sun-glare were reduced, that likely buffered large fluctuations in controlled atmosphere concentrations. Indeed, other authors have documented that the method of application, the type of facility and/or temperature/sunshine fluctuations can cause concentration fluctuations and uneven distribution of inert and toxic gases in treated stores, chambers and transport vehicles [11,44,71,72]. Regarding the importance of the necessity of an even concentration distribution of nitrogen in the treated chamber, Sakka et al. [44] stressed that: "pest location is a crucial parameter that may determine the insecticidal effect of nitrogen. In our study, we saw that despite variations in insect survival in the different locations, some of the insects that survived we found in the vials that had been placed in the "heart" of the pallet. This is somehow expected, as nitrogen penetrates into the pallet from the outside to the inside, and thus, "oxygen nests" can be created in the internal part of the product mass." From this point of view, it would be interesting to compare our results (concerning the stability and concentration variations of a controlled hypoxic atmosphere) with experiments carried out in small silos located outside shielded and tempered spaces, in the future experiments.

Concurrently with the validation of nitrogen filling and concentrations maintenance in the silos, bio tests were placed in the upper and lower parts of the silos. This is because our previous work [37] revealed that there might be differences in the biological efficacy achieved between the top and bottom of the silo when filling silos by nitrogen from top to bottom, and using very short exposures. However, in the current validation experiments, it was shown that both exposure regimes (11 and 21 days) resulted—under given environmental conditions—in 100% control of all developmental stages of *C. chinensis* and *S. granarius* at both the top and bottom of the treated silos. Thus, these tests yielded optimistic results, acceptable for the use of relatively short exposure times of controlled atmospheres in small silos. On-site nitrogen production using a swing-pressure-generator is economically challenging; especially in the current global energy crisis. Thus, the energy savings made possible by short exposure times, and thus reduced nitrogen production needs, is a very important and timely result of this work for practice. However, we believe that the results obtained for *C. chinensis* and *S. granarius* still need to be validated for other species. Indeed, there is an experimentally supported suspicion that some species, such as *T. granarium,* may be highly tolerant to hypoxic [44,46] and hyper-carbic [46] atmospheres. To control resistant species such as *T. granarium*, it will likely be necessary to increase the length of exposure, manipulate temperature/pressure [48,73], or use a combination of stressors such as radiation and controlled atmospheres [29]. Other aspects that merit

future research attention may be effects caused by differences in the sensitivity of different geographic strains of pests to anoxic and hypoxic atmospheres. The differences in the tolerance of different strains of pests to controlled atmospheres has been noted by Adler [54] and Conveyers and Bell [56], who reported differences in the responses to hypoxia of *S. granarius* populations from different countries and geographic regions.

5. Conclusions

Hypoxic or anoxic atmospheres belong among promising and environmentally acceptable methods of effective stored product pest control, both for farmers and for international phyto-quarantine services [16,74,75]. Moreover, they have the potential for the effective control of pest populations resistant to traditional fumigants, such as phosphine. In this paper, data were presented regarding both the laboratory and field efficacy of hypoxic atmospheres on different developmental stages of two primary key pest species (*C. chinensis* and *S. granarius*) with intra-grain immature development. The laboratory data indicated that 10–20 days' exposure resulted in 100% control of all developmental stages of *C. chinensis* and *S. granarius* when nitrogen concentrations was maintained between 99% and 100%, under defined temperature and humidity. Hypoxic nitrogen concentrations of 99% had even higher efficacy than completely anoxic atmospheres in the laboratory conditions tested. However, the 95% nitrogen concentration atmosphere did not result in 100% control of both species at the exposures given, even under controlled laboratory conditions for exposures of up to 20 days. Experimental validation in the silos, located indoors without large temperature fluctuations and direct sunshine-warming effects, showed that a targeted hypoxic concentration above 99% could be well achieved within 1 day of nitrogen initial purging, and stably maintained for the consequent 10–20 days. Bio tests indicated that both exposure regimes (10 + 1 and 20 + 1 days) resulted in 100% control of all developmental stages of *C. chinensis* and *S. granarius* under the given environmental conditions. Therefore, the use of nitrogen atmospheres in smaller silos for relatively short exposure periods can be considered a promising effective method for controlling *C. chinensis* and *S. granarius* species in small silos equivalent to the capacity of a standard shipping container. Thus, the results are of interest both for the protection of stored commodities on small farms, and for commercial organizations storing/treating separately a supply of commodities corresponding to the size of standard shipping containers. However, it is recommended to extend the tests and validations to other pest species and geographical strains/populations as they may have different sensitivities [56,66].

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